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The effect of *Dermatophagoides pteronyssinus* group 7 allergen (Der p 7) on dendritic cells and its role in T cell polarization

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ABSTRACT

The innate signaling pathway for Th2 immunity activated by inhaled allergens has not been well defined. Dendritic cells (DCs) can use their innate pattern-recognition Toll-like receptors and C-type lectin receptors to generate innate immunity and influence adaptive responses. The aim of this study was to investigate the glycoform of *Dermatophagoides pteronyssinus* group 7 allergen (Der p 7) and its functional interaction with dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN). Both bone marrow-derived dendritic cells (BMDCs) derived from mice and monocyte-derived DCs (MDDCs) derived from human peripheral blood mononuclear cells (PBMCs) were used to investigate the function of Der p 7. Dendritic cells derived from THP1 were used to investigate the glycol form of Der p 7. Der p 7 interacted with DC-SIGN as recognized by immunoprecipitation and glycoprotein staining, and its function was partially inhibited by deglycosylation. When BMDCs were cultured with Der p 7, the secretion of IL-6 and gene expressions of IL-6, OX40 L and Jagged-1 were increased. IL4⁺/CD4⁺ T cells could be induced by Der p 7, however IFN- γ /CD4⁺ T cells were down-regulated. The effects of Der p 7 on DCs were down-regulated in the presence of DC-SIGN or TLR4 antibodies and deglycosylation. rDer p 7 enhanced the DC differentiation of CD80 and CD86. The effects of Der p 7 pulsed-MDDCs on IL4⁺/CD4⁺ and IFN- γ /CD4⁺ T-cell differentiation of human PBMCs were significantly increased after Der p 7 stimulation and decreased by DC-SIGN antibody inhibition. In conclusion, the Der p 7 is a glycoprotein and its function was partially through glycan binding. BMDCs could be activated by Der p 7 through TLR4 and DC-SIGN, and followed by the expression of OX40 ligand (OX40L) and Jagged-1, the expressions of IL4⁺/CD4⁺ cells were enhanced. These findings identify a previously unrecognized function of Der p 7 and establish a link between innate TLR4/C-type lectin receptors and adaptive Th2 immunity.

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1. Introduction

Approximately 30% of the general population suffers from allergic diseases. Despite the fact that the symptoms of allergic diseases vary in complexity, severity and organ manifestation, the socioe-

conomic burden has continued to rise quite dramatically in recent years (Lai et al., 2002). The risk of developing an allergy is directly related to the acquisition of immediate hypersensitivity to environmental allergens (Tsai et al., 2002).

There are many environmental allergens such as dust mites, food, animal dander, insects and pollen, of which dust mites are one of most important. A total of 23 mite allergens have been defined (Banerjee et al., 2014), and the group seven allergens of *D. pteronyssinus* (Der p 7) and *D. farinae* (Der f 7) are considered to be major allergens because 50% of mite atopic patients have IgE antibodies to these allergens (Shen et al., 1996; Shen et al., 1997). Der p 7 is a 215 amino acid protein with a molecular weight of approximately 22 KDa, which has been reported to cause IgE immune responses in the majority of allergic patients (Shen et al., 1996).

In mammals, signaling of bacterial lipopolysaccharide (LPS), a major component of the cell wall of Gram-negative bacteria, pro-

Abbreviations: DCs, dendritic cells; Th2, T helper type 2; Der p 7, *Dermatophagoides pteronyssinus* group 7; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; BMDCs, bone marrow-derived dendritic cells; MDDCs, monocyte-derived dendritic cells; TLR4, Toll-like receptor 4; LPS, lipopolysaccharide; MD-2, myeloid differentiation-2; CLRs, C-type lectin-like receptors; PVDF, polyvinylidene difluoride; ELISA, Enzyme-linked immunosorbent assay; GM-CSF, Granulocyte-macrophage colony-stimulating factor.

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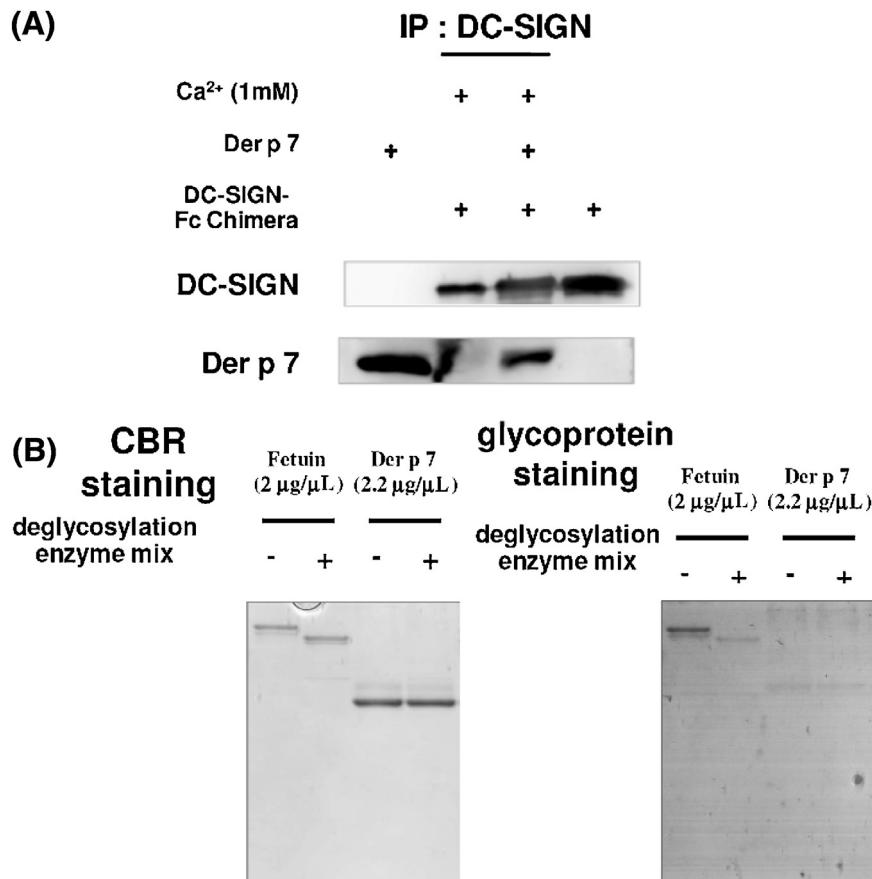


Fig. 1. The interaction of Der p 7 and DC-SIGN and glycoprotein identification. (A) Determination of the interaction between DC-SIGN and Der p 7 by co-immunoprecipitation assays. Purified DC-SIGN-Fc chimera proteins, recombinant Der p 7 proteins combined with anti-DC-SIGN anti-Der p 7 antibody were subjected to immunoprecipitation, followed by immunoblotting with the indicated antibodies. (B) Der p 7 was treated with or without deglycosylation enzyme mix, followed by SDS-PAGE, and the gels were performed by Coomassie Brilliant Blue R-250 staining and glycoprotein staining. Fetuin served as a positive control.

ceeds through TLR-4. Despite reports of a close proximity between TLR4 and LPS, TLR4 may not bind with LPS directly but may require the presence of the extracellular adaptor protein myeloid differentiation-2 (MD-2), which is bound to its ectodomain. MD-2 forms a stable complex with TLR4 on the cell surface, and has been implicated in LPS recognition (Thomas et al., 2010). The group 2 allergen of *D. pteronyssinus* has been identified as a MD-2 related lipid recognition protein (Trompette et al., 2009). Recent reports have indicated the similarity of the structure and function of Der p 2 and MD-2 in a MD-2 knockout murine model, the structural fold most compatible with human as well as murine MD-2 sequences (Trompette et al., 2009). Der p 7 is structurally homologous to human bactericidal permeability increasing protein/LPS binding protein (LBP), which may interact with TLRs binding with LPS and other bacterially derived lipid ligands (Mueller et al., 2010). It is feasible that Der p 7 may play a role in LPS recognition through TLRs (Thomas et al., 2010).

Dendritic cells (DCs) are professional antigen presenting cells which are key regulators of immune responses (Banchereau and Steinman 1998). DCs efficiently sample their microenvironment for antigens through a wide range of receptors, of which C-type lectin-like receptors (CLRs) appear to be of paramount importance in the recognition and uptake of glycoprotein antigens (Banchereau et al., 2000). Many CLRs have been identified to have an effect on DC function mainly as antigen uptake receptors, including mannose receptor (MR, CD206), DC-SIGN (CD209) and DEC-205 (CD205). nDer p 2 has been reported to be glycosylated and to have an effect on DC activation through DC-SIGN in addition to TLR4, and the

structure of Der p 7 has been reported to be similar to the innate immune protein LBP. Although Der p 7 has been reported to polarize Th2 cells from peripheral mononuclear cells (PBMCs), the mechanism of Th2 polarization has yet to be clarified. OX40 L and Jagged-1 have been reported to be important ligands for Th2 cell activation in patients with asthma (Okamoto et al., 2012; Kaur and Brightling 2012). Therefore, the aim of this study was to investigate whether the mechanism of Der p 7 on Th2 cell polarization is through DCs via OX40 L and Jagged-1.

2. Materials and methods

2.1. Immunoprecipitation (IP) and western blot analysis

Recombinant Der p 7 (INDOOR Biotechnologies, Virginia, USA) and DC-SIGN-Fc fusion proteins were used for immunoprecipitation assays. Der p 7 Ab (WH9 provided by Prof. Horng-Der Shen, Department of Medical Research and Education, Taipei Veterans General Hospital) and DC-SIGN Ab (R&D Systems, Minneapolis, USA) were added to the binding reaction mixtures, respectively, overnight at 4 °C. Immune complexes were precipitated with protein G-Sepharose (Sigma, MO, USA) according to the manufacturer's instructions and washed with phosphate-buffered saline (PBS) three times. The precipitated proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, MA, USA) pre-activated in methanol. The blots were then washed three times with PBS containing 0.05% Tween-20 (PBST). The washed blots were immersed in a solution of

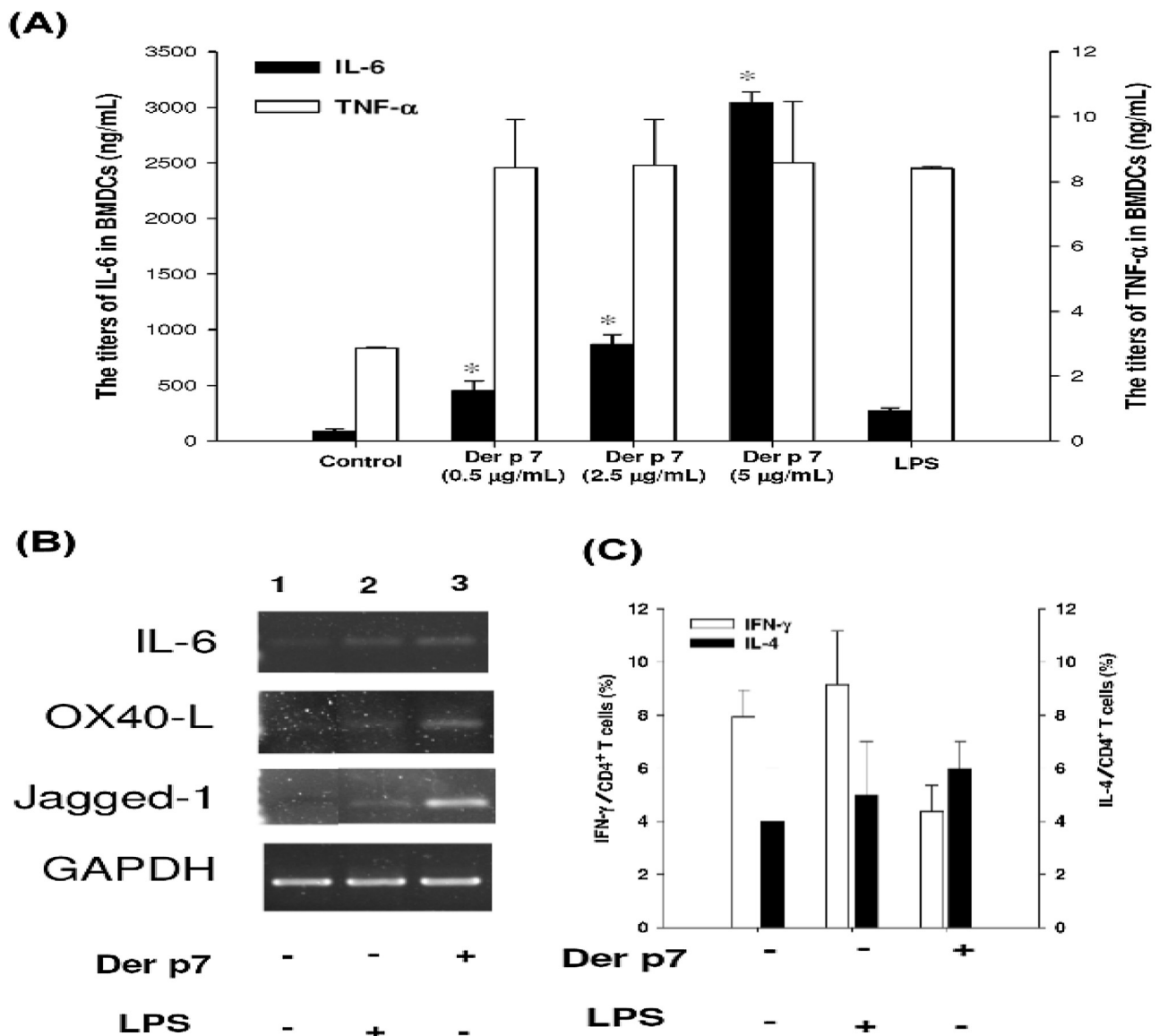


Fig. 2. The effect of Der p 7 on cytokine secretion and mRNA expression of OX40 L and Jagged-1 in BMDCs, and the effects of Der p 7-pulsed DCs on IL-4 $^{+}$ /CD4 $^{+}$ and IFN- γ $^{+}$ /CD4 $^{+}$ T-cells from BMDCs. (A) BMDCs (5×10^5 cells/sample) in a 24 wells were treated with serial doses of Der p 7 (0, 0.5, 2.5, 5 μ g/mL) for 24 h. The supernatant was collected and measured by ELISA. The negative control was medium only and the positive control was LPS (10 ng/mL). (B) mRNA was isolated by Trizol and analyzed by RT-PCR. The BMDCs (5×10^5 cells/sample) were treated with LPS (10 ng/mL) or Der p 7 (5 μ g/mL) for 24 h in a 24-well plate. The expressions of OX40L, IL-6 and Jagged-1 were measured by RT-PCR. The results were mean $\pm$ SD from three experiments. * $p < 0.05$ compared with the control. (C) Splenocytes from mice were co-cultured with BMDCs (2×10^6 cells) and treated with Der p 7 (5 μ g/mL) for 24 h. The production of IL-4 and IFN- γ in CD4 $^{+}$ T cells was measured and analyzed by flow cytometry. A total of 10,000 events were acquired for analysis. The data are presented as percentages of IFN- γ /CD4 $^{+}$ and IL-4/CD4 $^{+}$ in CD4 $^{+}$ T cells ($n = 3$).

primary antibody diluted in PBST and incubated at 4°C for 3 h. The blots were then washed three times with PBST. The secondary antibody of goat anti-mouse IgG horseradishperoxidase-conjugated (Life technologies, CA, USA) diluted in PBST was then added. After incubation at room temperature for 2 h, the blots were washed three times with PBST. The bands were stained by adding the substrate solution of enhanced chemiluminescence reagent (Millipore, MA, USA) to reveal the target proteins and analyzed by an Auto-Chemi System (LAS 3000, Fujifilm).

2.2. Glycoprotein staining

Fetuin and recombinant protein rDer p 7 were treated with or without deglycosylation enzyme mix (NEW ENGLAND Bio-Labs, UK), and were applied to SDS-PAGE at the indicated protein

concentration. After electrophoresis, the gel was stained with a glycoprotein staining kit (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions, and subsequently stained with Coomassie Brilliant Blue R-250. Fetuin served as a positive control.

2.3. Mouse bone marrow-derived dendritic cell (BMDC) preparation

Male C57BL/6 mice (6–8 weeks old) were purchased from the National Laboratory Breeding Research Center in Taiwan and housed in a specific pathogen-free barrier environment at Taichung Veterans General Hospital (TCVGH), Taichung, Taiwan. Mice were kept at a relatively humidity of $55 \pm 5\%$, a constant temperature of $23 \pm 1^\circ\text{C}$ and under a regular of day and night rotation (light: dark = 12 h: 12 h). The Animal Experimentation Ethics Committee of

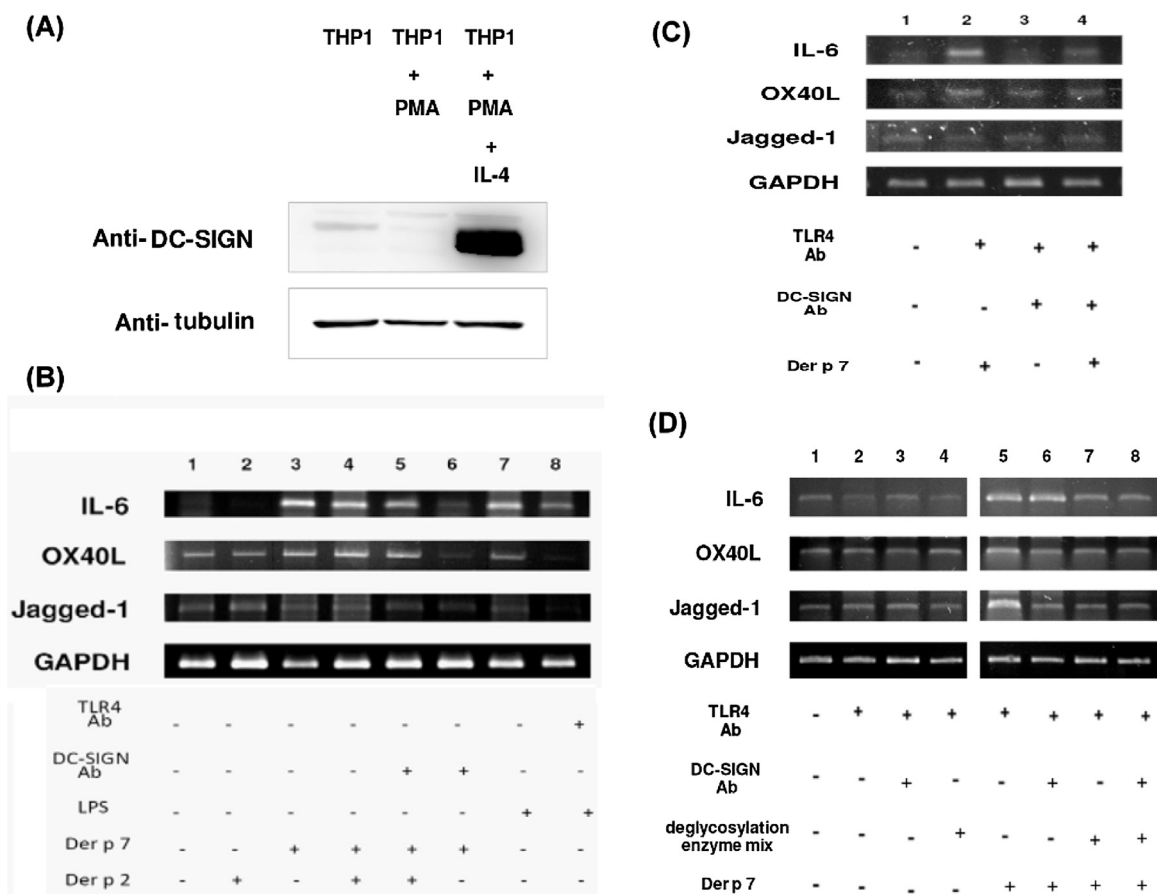


Fig. 3. Effects of DC-SIGN and TLR4 antibodies on the modulation of cytokine gene expressions from Der p 7-pulsed DCs. (A) Expression of DC-SIGN was determined by Western blot in THP-1 cells. THP1 derived DCs were used in the following experiments. (B) Der p 7-pulsed DCs (5×10^5 cells/sample) in a 24 wells were cultured with or without TLR4 or DC-SIGN antibodies, followed by gene expression analysis. (C) Der p 7-pulsed DCs (5×10^5 cells/sample) in a 24 wells were incubated with TLR4 or DC-SIGN antibodies followed by gene expression analysis. (D) Der p 7 treated with or without deglycosylation enzyme mix was used to pulse DCs (5×10^5 cells/sample) in a 24 wells in the presence or absence of TLR4/DC-SIGN antibodies by gene expression analysis.

the Taichung Veterans General Hospital approved the experimental procedures. Bone marrow from the femur and tibia of C57BL/6 mouse was placed in a 60 mm dish and washed with 70% alcohol for 1 min. The bone marrow cells were flushed using a 25-gauge needle and transferred into a fresh dish with RPMI 1640. The red blood cells were lysed with ammonium chloride and washed twice with PBS. The cells were maintained in RPMI 1640 media containing 10% Fetal Bovine Serum and 1% penicillin/streptomycin supplemented with recombinant murine GM-CSF (10 ng/mL) (R&D Systems Inc., MN, USA) and IL-4 (10 ng/mL) (R&D Systems Inc., MN, USA). The cultures were fed with fresh medium containing Granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 by gently swirling the plate. On day 7, the cells were transferred to 12-well plates with 2×10^5 cells/well for the experiments. CD11c⁺/MHC class II⁺ cells were confirmed by flow cytometry (>90%).

2.4. BMDC treatment and harvest

BMDCs were placed on 12-well plates for 24 h. The cells were pre-treated with LPS (Sigma, MO, USA) (10 ng/mL) and DC-SIGN antibody (R&D Systems Inc., MN, USA) (5 μ g/mL) for 2 h and then stimulated with Der p 7 (5 μ g/mL). For gene analysis, the cell lysate was collected using a High Pure RNA Isolation Kit provided by Roche Diagnostics (Mannheim, Germany) for mRNA expression analysis. For enzyme-linked immunosorbent assay analysis, culture supernatant was collected from the 12-well plates after 24 h of stimulation.

2.5. Determination of gene expressions by RT-PCR

Total RNA was extracted from 1×10^6 DCs using a High Pure RNA Isolation Kit provided by Roche Diagnostics. RNA quality and quantity were evaluated by ultraviolet spectrophotometry and agarose gel electrophoresis. cDNA was generated from 1 μ g of total RNA for each sample using a Reverse Transcriptase System with oligo-dT primers (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions. Gene expression was determined by PCR. Gene-specific forward and reverse primer sequences (all 5'-3') are listed in supplementary Table.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Commercially available ELISA kits were used to determine cytokine levels in the cell culture supernatants. The cell supernatants were collected after Der p 7 had been treated with or without LPS for the indicated times, and the levels of IL-6 and TNF- α were quantified by ELISA. The detection limit was 30 pg/mL. Plates were read on a SpectraMax M2 microplate reader (Molecular Devices, CA, USA) and analyzed with SOFTmax analysis software (Molecular Devices, CA, USA). The means of triplicate ELISA values for each of the dose relationships among these protein expressions were calculated by linear regression.

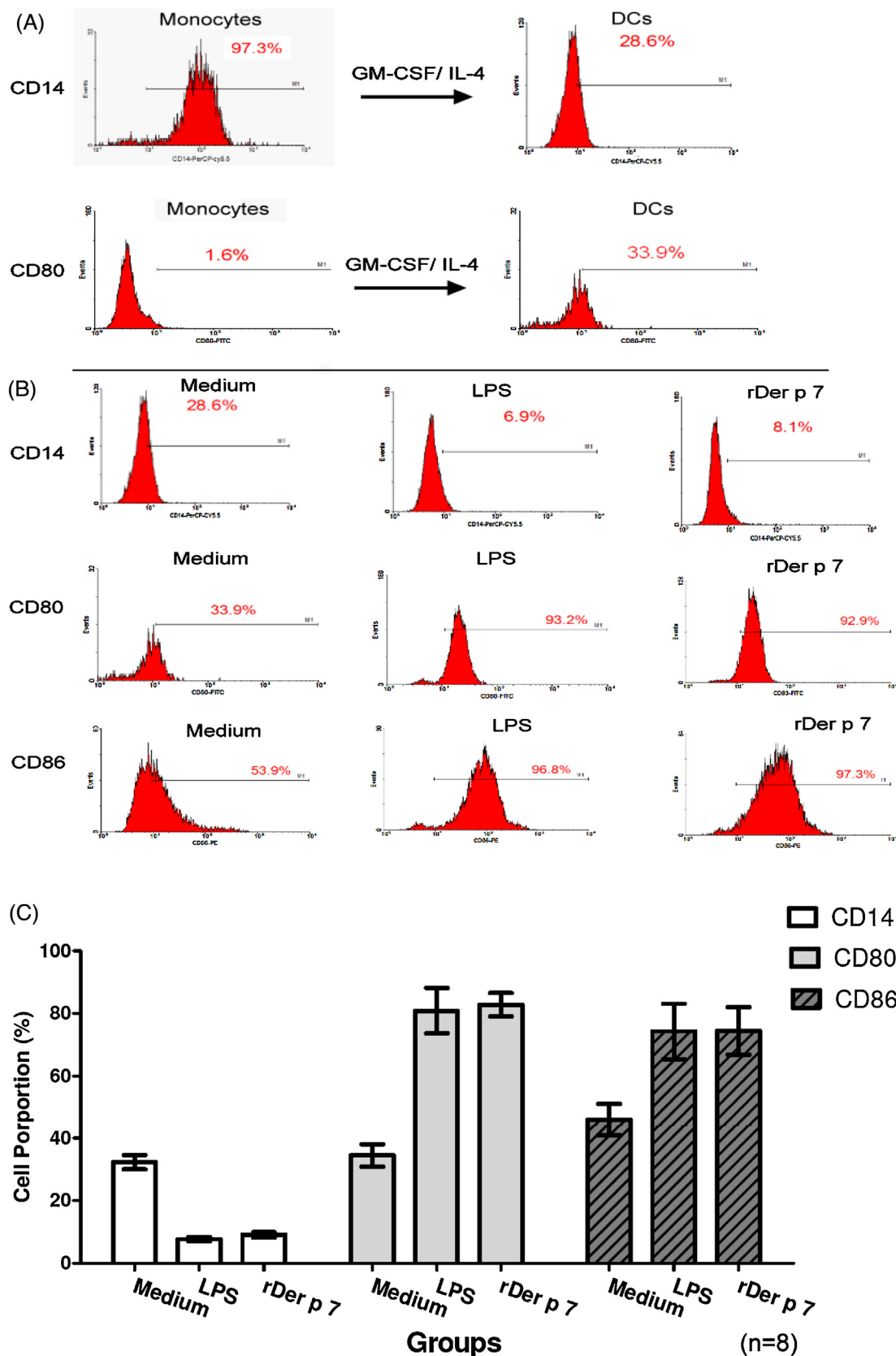


Fig. 4. The allergen Der p 7 induced the expression of surface markers on DCs derived from monocytes. (A) PBMCs were isolated from the participants and the monocytes were purified by CD14⁺ microbeads as described in Methods. The differentiation of DCs derived from monocytes was stimulated with GM-CSF/IL-4, and the surface markers of the monocytes were confirmed by CD14⁺ staining and flow cytometry. (B) After differentiation, the MDDCs (5×10^5 cells/sample) in a 24 wells treated with LPS or rDer p 7 for 3 days. Expression of surface markers (CD80 and CD86) was analyzed by flow cytometry. The values shown in the flow cytometry profiles are the gated% and the mean fluorescence intensity indexes. A total of 10,000 events were acquired for analysis. Cells expressing the antigen of interest are indicated within each histogram. (C) A total of eight allergic patients were recruited for observing the changes of CD14, CD80 and CD86 surface markers after the LPS or rDer p 7 treatments for 3 days.

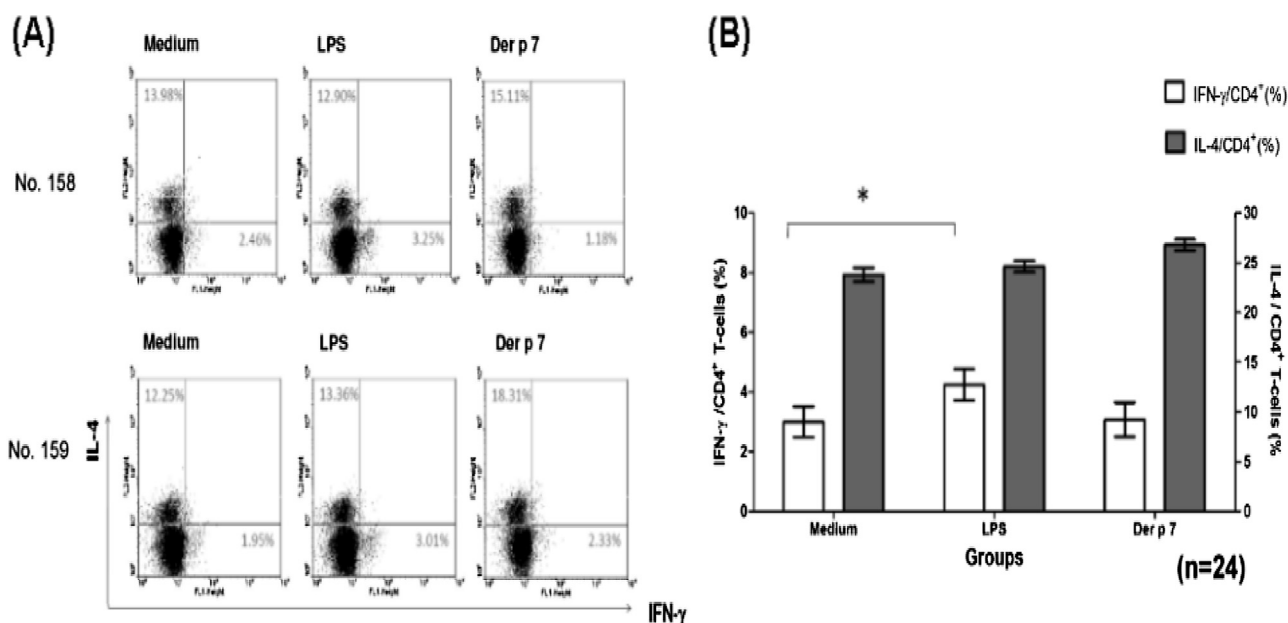


Fig. 5. Effect of Der p 7 on the T-cell subpopulation of PBMCs. (A) The cells were stimulated with PMA (50 ng/mL), ionomycin (1 μ g/mL) and GolgiStop for intracellular staining. The cells were stained with peridinin chlorophyll-a protein (Per-CP)-conjugated anti-human CD4 monoclonal antibody, fluorescein isothiocyanate-conjugated anti-human IFN- γ monoclonal antibody and R-phycoerythrin-conjugated anti-human IL-4 monoclonal antibody. The cells from human PBMCs stimulated with Der p 7 were analyzed by flow cytometry. (B) The percentage of positive cytokine-stained cells (IFN- γ or IL-4) in CD4⁺ lymphocytes from the human PBMCs. A total of 10,000 events were acquired for analysis. Data are expressed as percentages of mean $\pm$ standard deviation (n = 24). *p < 0.05 compared to the medium group.

2.7. Cell cultures of human THP-1 monocytic leukemia and Jurkat T cell lines

The human monocytic leukemia cell line THP-1 was kindly provided by Dr. Meei Ling Sheu (College of Life Sciences, National Chung Hsing University, Taiwan), and was cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and 1% penicillin-streptomycin. DC-SIGN expression was induced by treatment with or without phorbol myristate acetate (PMA) (20 ng/mL) and IL4 (20 ng/mL) for 5 days. After induction, PMA/IL-4 stimulated THP-1 cells were treated with various stimuli including LPS (1 ng/mL), Der p 2 (5 μ g/mL) or rDer p 7 (5 μ g/mL). The Der p 2 was purchased from commercial company (product code: LTN-DP2-1, INDOOR Biotechnologies, Virginia, USA). The Der p 2 was purified by multi-step chromatographic purification and the content of endotoxin was lower than 0.03 EU/ μ g. After 24 h, the supernatants were discarded and the cells were then co-cultured with Jurkat T cells for another 24 h. To block the allergen-mediated effect through TLR4 or DC-SIGN, the cells were cultured in the presence of anti-TLR4 (0.5–5 μ g/mL, Santa Cruz Biotechnology, California, USA) or anti-DC-SIGN Abs (10 μ g/mL, R&D Systems Inc. MN, USA) for 2 h before allergen stimulation. Jurkat T cells were obtained from the Food Industry Research and Development Institute (FIRDI, Hsin Chu, Taiwan) and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (complete medium).

2.8. Preparations of human peripheral blood mononuclear cells (PBMCs) and monocyte-derived dendritic cells (MDDCs)

The PBMCs from the study participants (including twenty-four *D. pteronyssinus* allergic patients and three healthy controls) were obtained from the Allergy Clinic at Taichung Veterans General Hospital, Taichung, Taiwan. This study was approved by the Institutional Research Board of Taichung Veterans General Hospital (IRB TCVGH No. CE13123), and informed written consent was obtained from each participant. Sensitivity to mite *D. pteronyssinus* was

determined by mite specific IgE measurement using a Pharmacia CAP System (Uppsala, Sweden). A titer of antigen specific IgE higher than 0.35 kU/L was considered to be a positive result. All of the donor's blood products were negative for common blood-borne pathogens as detected by standard assays, and PBMCs were isolated from whole heparinized blood by Ficoll-Paque density centrifugation (GE Healthcare, Waukesha, USA) as the manufacturer's instructions. The resulting PBMC layer was recovered and washed twice with PBS. The total number of cells was calculated in each sample using a hemacytometer followed by CD14⁺ microbeads for monocyte purification following the manufacturer's instructions (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The cells were then cultured in RPMI 1640 medium supplemented with GM-CSF (50 ng/mL) and IL-4 (10 ng/mL) for 7 days. MDDCs derived from GM-CSF/IL-4 stimulated monocytes were confirmed by flow cytometry using anti-CD80 and anti-CD86 monoclonal antibodies. MDDCs were treated with Der p 7 with/without TLR4 Abs/DC-SIGN Abs for 24 h, following co-cultured with autologous PBMCs at a DC/PBMC ratio of 1:10 by cell co-culture plate (CBA-160, CytoSelect™ 24-well Cell Co-Culture System, Cell Biolabs, Inc., San Diego, CA, USA) for 3 days followed by IFN- γ /CD4⁺ and IL-4/CD4⁺ cell analysis.

2.9. Cell surface antigen and intracellular cytokine staining

After the incubation period, cells were washed twice and resuspended with PBS. The cells were stimulated with phorbol 12-myristate 13-acetate (PMA) (50 ng/mL), ionomycin (1 μ g/mL) and GolgiStop (Cytofix/Cytoperm Plus PharMingen, San Diego, Calif, USA) for four hours and then washed twice with PBS. The PBMCs were resuspended to a density of 5×10^6 cells/mL and 100 μ L aliquots of the cell suspension were distributed into tubes for staining. For the cell surface staining, cells were incubated with CD4-PerCP (BD Biosciences) at room temperature for 30 min in the dark and then washed. Cells were permeabilized and fixed with Cytofix/Cytoperm at room temperature for 30 min stained with

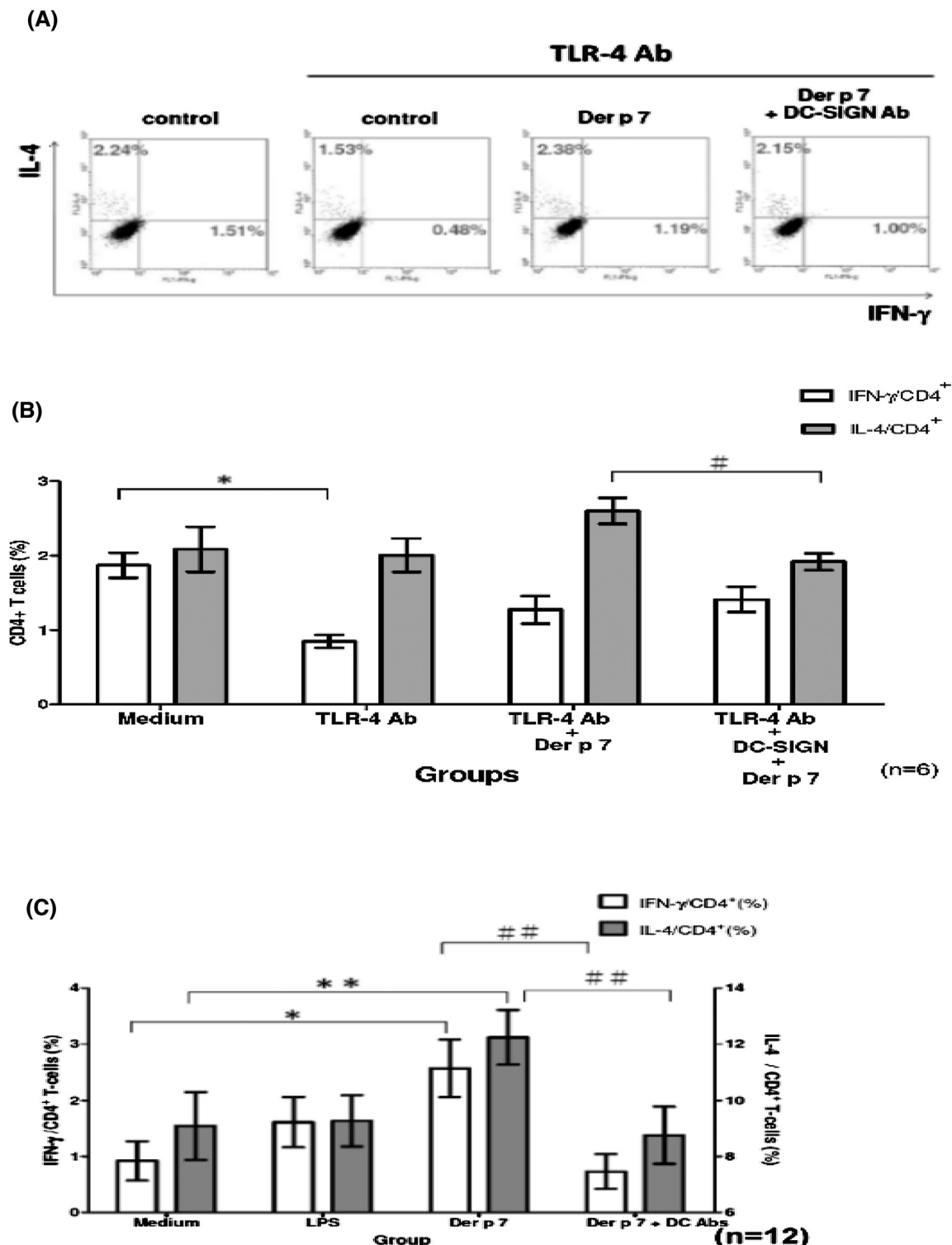


Fig. 6. Effect of Der p 7 pulsed-MDDCs on the lymphocyte subpopulation. (A) The dendritic cells derived from THP-1 cells were co-cultured with Jurkat T cells. The co-culture ratio of DC/Jurkat T cells is 1:10. The cells were stimulated with PMA (50 ng/mL), ionomycin (1 μ g/mL) and GolgiStop for intracellular staining. Then, the cells were treated with Der p 7 (5 μ g/mL) and DC-SIGN Ab (5 μ g/mL) for 3 days followed by flow cytometry analysis. A representative data of cytometry analysis was presented. Medium only was used as the negative control. (B) A total of 10,000 events were acquired for analysis. Data are expressed as percentages of mean $\pm$ standard deviation (n = 6). *p < 0.05 compared to the medium group. #p < 0.05 compared to the DC-SIGN Ab treatment. (C) MDDCs (5 $\times$ 10⁵ cells/sample) derived from GM-CSF/IL-4-stimulated PBMCs were collected for co-culture with the PBMCs. The MDDCs (5 $\times$ 10⁵ cells/sample) were pulsed with Der p 7 in the presence or absence of DC-SIGN antibodies for 24 h, followed by co-culture with autologous PBMCs for 3 days. The T-cell subpopulations of IFN- γ /CD4⁺ and IL-4/CD4⁺ in CD4⁺ T cells were analyzed by flow cytometry. A total of 10,000 events were acquired for analysis. Data are expressed as percentages of mean $\pm$ SD (n = 12). *p < 0.05; **p < 0.01 compared to the medium group. ##p < 0.01 compared to DC antibody inhibition.

IL-4-PE, IFN- γ -FITC (BD Biosciences) and negative controls (corresponding IgG isotypes) at room temperature for 30 min and then washed. Cells were resuspended in 0.5 mL PBS and measured by Becton Dickinson flow cytometry (BD, San Jose, Calif, USA). A total of 10,000 events were acquired for each sample for analysis.

3. Results

3.1. Interaction with DC-SIGN and glycan identification of Der p 7

The interactions of Der p 7 and DC-SIGN were identified by using reciprocal immunoprecipitation(IP)-Western blot analysis. This study used Der p 7 and DC-SIGN Fc chimera, and the results showed that they interacted with each other to form a protein complex (Fig. 1A). This binding of DC-SIGN directly to Der p 7 was Ca²⁺ dependent. Fetuin is a glycoprotein containing sialylated N-linked and O-linked glycans that can be used as a positive control for endoglycosidase enzymes. The results showed that the glycans of fetuin could be digested in the presence of a deglycosylation enzyme mix (Fig. 1B). The glycan in Der p 7 was not obviously detected by Coomassie Brilliant Blue staining, however it could be identified by direct glycoprotein staining (Fig. 1B). The interaction of DC-SIGN and Der p 7 was identified by using a DC-SIGN Fc fusion protein immunoprecipitation assay.

3.2. Effect of Der p 7 on cytokine secretion and mRNA expression of OX40 and Jagged-1 in BMDCs

In order to examine the effect of Der p 7 activation in the animal model, DCs from the bone marrow of mice were isolated, cultured and examined with Der p 7. BMDCs were treated with Der p 7 at serial doses (0, 0.5, 5 μ g/mL) for 24 h and analyzed with ELISA. The results showed that the secretion of IL-6 was significantly increased by Der p 7 in a dose-dependent manner compared with LPS. The expression of TNF- α was not significantly increased by Der p 7 (Fig. 2A). The mRNA expressions in the BMDCs were analyzed by RT-PCR. The BMDCs were treated with Der p 7 or LPS, and the results showed that the expressions of IL-6, OX40 L and Jagged-1 were increased by Der p 7 or LPS. The expressions of OX40 L and Jagged-1 were more significantly induced by Der p 7 than by LPS (Fig. 2B). After mouse splenocytes had been co-cultured with Der p 7-pulsed BMDCs for three days, the expressions of IL-4 and INF- γ in CD4⁺ T cells were analyzed by flow cytometry. The results showed that IL4⁺/CD4⁺ T cells could be induced by Der p 7, however IFN- γ ⁺/CD4⁺ T cells were down-regulated (Fig. 2C). In contrast, IFN- γ ⁺/CD4⁺ T cells could be induced by LPS.

3.3. Effect of DC-SIGN and TLR4 antibodies on cytokine gene expression from Der p 7-pulsed DCs

DCs transformed from THP-1 were obtained after culture with PMA and IL-4, and the expression of DC-SIGN was found to be up-regulated after DC transformation (Fig. 3A). Der p 7- pulsed DCs were cultured with or without TLR4 or DC-SIGN antibodies followed by gene analysis, and the results showed that the expression of IL-6 induced by LPS was inhibited by TLR4 antibodies. The expressions of IL-6, OX40 L and Jagged-1 were increased after Der p7 treatment, but were inhibited by DC-SIGN antibodies (Fig. 3B). When the cells were cultured with Der p 7 in the presence of DC-SIGN and TLR4 antibodies, the expressions of IL-6 and OX40 L were down-regulated (Fig. 3C). No significant differences were observed of Jagged-1 expression when cells cultures with Der p 7 in the presence of DC-SIGN and TLR4 antibodies (Fig. 3C). The effect of Der p 7 was decreased to a similar extent as that after deglycosylation (Fig. 3D).

3.4. Effect of Der p 7-induced surface marker expression on the DCs

The PBMCs were isolated from whole blood obtained from the participants. The monocytes were purified by CD14⁺ microbeads and analyzed by flow cytometry. The differentiation of DCs derived from monocytes was stimulated with GM-CSF/IL-4 for 7 days, and the surface markers of the monocytes were confirmed by CD14⁺ staining and flow cytometry. After the CD14⁺ microbead purification, the results showed that 97.3% of the cells had a CD14 surface marker expression (Fig. 4A). After DC differentiation, this level fell to 28.6% (Fig. 4A). In addition, the surface marker expression of CD80 increased from 1.6% to 33.9% (Fig. 4A). After differentiation, a representative data showed that the DCs were apparently increased after the LPS or rDer p 7 changes in CD80 and CD86 expressions (Fig. 4B). A total of eight allergic patients were recruited for observing the changes of CD14, CD80 and CD86 surface markers after the LPS or rDer p 7 treatments for 3 days. It showed that there was obviously decreased expression of CD14 surface marker, otherwise there were significantly increased expressions of CD80 and CD86 surface markers (Fig. 4C).

3.5. Effect of Der p 7 induction on IL4⁺/CD4⁺ and IFN- γ ⁺/CD4⁺ T cell differentiation of human PBMCs

Human PBMCs from *D. pteronyssinus* allergic patients and three healthy controls were collected and pretreated with Der p 7 or LPS for 3 days. The leucocytes were then cultured with PMA/ionomycin for 4 h before analysis of the CD4⁺ cells producing the TH1-type cytokine IFN- γ ⁺ and the TH2-type cytokine IL-4. Two representative data from two allergic patients (No. 158 & No. 159) were presented in Fig. 5A; furthermore, there were not apparent change in the healthy controls after the Der p 7 or LPS treatment (data not shown). The percentage of IFN- γ ⁺/CD4⁺ T cells was significantly increased after LPS treatment compared with the medium group ($p < 0.05$) (Fig. 5B). No significant differences were found in IFN- γ ⁺/CD4⁺ and IL4⁺/CD4⁺ T cells between the medium and Der p 7 groups.

3.6. Effect of Der p 7 pulsed-MDDCs on IL4⁺/CD4⁺ and IFN- γ ⁺/CD4⁺ T cell differentiation of human PBMCs I

The human DCs derived from THP-1 cells were co-cultured with Jurkat T cells for 24 h. The DCs were then treated with Der p 7 (5 μ g/mL) for 3 days in the presence of TLR4 antibodies (5 μ g/mL) with or without DC-SIGN antibodies (5 μ g/mL) followed by cytometry analysis. A representative data of cytometry analysis was presented in Fig. 6A. The results showed that the percentages of IFN- γ ⁺/CD4⁺ T cells were decreased by TLR4 antibody inhibition (Fig. 6B). When the Jurkat T cells were co-cultured with the DCs, the percentage of IL4⁺/CD4⁺ induced by Der p 7 could not be inhibited by TLR-4 antibodies, but could be inhibited by DC-SIGN antibodies (Fig. 6B). The MDDCs from the participants were co-cultured with PBMCs, and then treated with LPS and Der p 7 with or without DC-SIGN Ab for 24 h. The percentages of IL4⁺/CD4⁺ and IFN- γ ⁺/CD4⁺ in the T cells were then analyzed, and the results showed that IL4⁺/CD4⁺ and IFN- γ ⁺/CD4⁺ both significantly increased after Der p 7 stimulation ($p < 0.05$) (Fig. 6C). In the presence of DC-SIGN antibodies, a significant decrease was found in the percentages of IL4⁺/CD4⁺ and IFN- γ ⁺/CD4⁺ compared with the Der p 7 group ($p < 0.05$) (Fig. 6C).

4. Discussion

Der p 7 is an important mite allergen which can elicit strong IgE Ab and T cell responses in patients with mite allergy (Hales

et al., 2000; Chou et al., 2011). However, its role in mite sensitization has not been characterized to date. In this study, we focused on the role of Der p 7 in DC activation and cytokine production through the interaction of DCs and T cells. The first stage we wanted to comprehend whether the DCs from the bone marrow of mice could be activated by Der p 7, the results showed the mRNA expression of inflammatory cytokine IL-6 was significantly increased via OX40 L and Jagged-1 ligands. The purpose in this experiment was to observe whether the BMDCs could be activated by allergen Der p 7 via important ligands (via OX40 L and Jagged-1) for Th2 cell activation. Although the mRNA expression of Th2-related cytokines as IL-4, IL-5, and IL-13 was not investigated, the expression of IL-4 and IFN- γ in CD4⁺ T-cells were analyzed by flow cytometry, and it showed IL-4⁺/CD4⁺ could be induced by Der p 7 by the animal experiments.

Our results showed that Der p 7-pulsed MDDCs could up-regulate IL4⁺/CD4⁺ T cells, and the effect of Der p 7 could be inhibited by antibodies against TLR4 and DC-SIGN. The Der p 7 is a glycoprotein and its function was partially through glycan binding, which could be inhibited after deglycosylation. We further confirmed glycan binding through immunoprecipitation-Western blot analysis and carbohydrate staining. House dust mite is the most common aeroallergen worldwide, and its extract has been shown to stimulate DCs, elicit sensitization and trigger Th2-dependent airway inflammation (Huang et al., 2011). Our results showed that Der p 7 could activate DCs and trigger Th2 cytokine production, indicating that Der p 7 is an allergen in dust mites which can contribute to Th2-mediated allergic inflammation.

DCs use their innate pattern-recognition TLRs, C-type lectin receptors, and DC-SIGN to generate innate immunity and influence adaptive immunity. DC-SIGN recognizes complex glycan structures on various pathogens through C-type carbohydrate-recognition domains to facilitate the endocytosis of pathogens (Barrett et al., 2009). Glycans have been reported to be present in plant allergens and to act as potent Th2 adjuvants (Shreffler et al., 2006). Ara h 1, the immunodominant peanut allergen, contains glycan which is recognized by DC-SIGN, and has been reported to confer human MDDCs with the capacity to generate IL-4 and IL-13 producing CD4⁺ T cells from naïve CD45RA⁺ T cells (Barrett et al., 2011). The glycan of the *Schistosoma mansoni* egg antigen (SEA) has been reported to elicit Th2 cytokines and antigen-specific IgE in Balb/c mice, and this activity has been reported to be lost with SEA deglycosylation (Okano et al., 1999, 2001). In the current study, the glycan component in Der p 7 was confirmed by immunoprecipitation and carbohydrate staining. DC-SIGN was found to bind directly to Der p 7 through immunoprecipitation-Western blot analysis, and the presence of carbohydrate staining in Der p 7. Similarly, Der p 2 has been reported to bind to DC-SIGN through glycosylation epitope (Hsu et al., 2010).

TLR4 has been reported to recognize LPS on bacteria through MD-2 and LPS binding protein (Thomas et al., 2005; Trompette et al., 2009). Der p 7 has been reported to be structurally related to a protein in the TLR pathway similar to LBP (Mueller et al., 2010). After binding to LPS and bacterially derived lipid ligands, Der p 7 can interact with LPS activated DCs. In this study, anti-TLR-4 Ab down-regulated DC activation when the DCs were stimulated with Der p 7 in conjunction with Der p 2 and LPS. These data suggest that Der p 2 and Der p 7 are important immunologic components and should be included in diagnosis and immunotherapy for mite allergy. So far, allergen function such as mite group 2 allergen has been comprehended the interaction with the innate immune system and generation of allergenicity. The results from this study, we suggest that there might be similarities between the group 2 and group 7 mite allergens of biological functions that involve binding of TLR-4 or DC-SIGN that both allergens might participate innate immune response into promoting allergenicity. Currently, OX40 L

and Jagged-1 are considered to be important ligands for Th2 cell regulation in patients with asthma (Okamoto et al., 2012; Kaur and Brightling 2012).

In conclusion, the current study showed that the biological function of Der p 7 on DC activation was significantly reduced with the absence of binding with DC-SIGN and TLR-4, which was associated with diminished expressions of OX40L, Jagged-1 and Th2 cytokine production. The effect of Der p 7 on DCs was inhibited not only by anti-TLR-4 but also by DC-SIGN antibodies, suggesting that glycan component also played a pathogenic role in DC activation. Der p 7-pulsed DCs could regulate the expressions of OX40 L and Jagged-1 and modulate T cell responses. In addition, IL4⁺/CD4⁺ cells could be enhanced and down-regulated by TLR4 and DC-SIGN antibodies. These findings may provide new therapeutic methods to prevent the allergy sensitization caused by Der p 7.

Conflict of interest

The authors declare no competing or financial interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.imbio.2016.04.002>.

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