Journal of Bacteriology and Virology 2014. Vol. 44, No. 4 p.311 – 316
http://dx.doi.org/10.4167/jbv.2014.44.4.311

Original Article

Panonychus citri Can Induce T-helper Type 2 Immune Responses via the Release of Thymic Stromal Lymphopoietin and IL-4

Jaechun Lee, Seong-Chul Hong, Young-Bae Chung, Hye-Sook Lee and Keun Hwa Lee*

The Environmental Health Center (Atopic Dermatitis and Allergic Rhinitis), Jeju National University School of Medicine, Jeju, Korea

Panonychus citri damages the leaves of citrus trees, causing defoliation, and induces T-helper type 2 (T_H2) immune responses (occupational asthma) via a hitherto unknown mechanism. This is a particular problem on Jeju Island, which is located to the south of the Korean peninsula. In this study, we show for the first time how *P. citri* induces T_H2 immunity. Exposure to *P. citri* induces the production of thymic stromal lymphopoietin (TSLP) by either basophils or $CD4^+$ T cells (it is not certain which), which results in the production of interleukin 4 (IL-4). IL-4 promotes the production of immunoglobulin E (IgE), which ultimately contributes to the process of allergic inflammation. Therefore, TSLP plays an important role in the *P. citri*-induced T_H2 immune response.

Key Words: *P. citri*, Occupational asthma, TSLP, IgE, IL-4.

INTRODUCTION

Under certain circumstances, the T-helper type 2 (T_H2) immune response can have deleterious effects, resulting in significant tissue injury or serious disease. One of these inappropriate and damaging immune responses is the IgE-mediated (type I) hypersensitivity reaction (allergic inflammation), which is induced by allergens (1).

Metazoan parasites and simple protein allergens induce a T_H2 immune response (allergic inflammation) (2~4). The citrus red mite, *Panonychus citri*, has been reported to damage the leaves of citrus trees, in some cases causing defoliation, on Jeju Island, which is located to the south of the Korean peninsula (5). In addition, Kim *et al*. have reported cases of *P. citri*-induced occupational asthma (IgE-mediated broncho-constriction) (5). The mechanism underlying this allergen-induced T_H2 immune response has not yet been studied.

Thymic stromal lymphopoietin (TSLP) is an interleukin (IL)-17-like cytokine that is involved in lymphocyte development (2, 6); it is also linked to the recruitment of T_H2 cells and allergic inflammation (2, 3, 6). Epithelial cells such as keratinocytes or basophils produce TSLP during the development of allergic disease, and TSLP leads to T_H2 cell recruitment, and ultimately allergic inflammation (2, 6, 7).

In this study, we show for the first time how *P. citri* can induce a T_H2 immune response. Exposure to *P. citri* causes the production of TSLP by either basophils or $CD4^+$ T cells (it is uncertain which), which in turn results in the pro-

Received: September 12, 2014/ Revised: October 7, 2014/ Accepted: October 10, 2014
*Corresponding author: Keun Hwa Lee, Ph.D. Department of Microbiology and Immunology, Jeju National University School of Medicine Jeju, 690-756, South Korea.
Phone: +82-64-754-8111, Fax: +82-64-705-2687, e-mail: yomust7@ jejunu.ac.kr
**This research was supported by the 2014 scientific promotion program funded by Jeju National University.

duction of IL-4. IL-4 stimulates the production of IgE, which ultimately contributes to the T_H2 immune response (allergic inflammation) (2, 8). Thus, TSLP plays an important role in the *P. citri*-induced T_H2 immune response.

MATERIALS AND METHODS

P. citri extract

P. citri were collected from the remains of naturally infected citrus orchards and homogenized in a Teflon pestle homogenizer with sterile phosphate-buffered saline (10 mM, pH 7.4). The homogenate was centrifuged at 15,000 rpm for 40 min. The resulting supernatant was filtered using a sterile membrane filter (0.2 μm, Microfiltration System, CA, USA). The filtered supernatants were used as a crude extract of *P. citri* and frozen at -70℃ until used.

Human peripheral blood mononuclear cell stimulation

Fresh human peripheral blood mononuclear cells (PBMCs) were isolated from healthy human blood specimens using density gradient centrifugation with Ficoll-Paque PLUS (GE Healthcare Bio-Sciences AB, 17-1440-02) and cultured (1×10^6/well) in 24-well plates at 37℃ in 5% CO_2 and in the presence or absence of *P. citri* extract (50 μg/well) in complete RPMI medium 1640. *P. citri*-stimulated PBMCs were harvested for evaluation of *Tslp* and *Il-4* gene expression by real-time PCR. Supernatants were collected and tested for the presence of TSLP, IL-4, and IgE (9).

Human $CD4^+$ T-cell stimulation

PBMCs were isolated from healthy human blood by density gradient centrifugation with Ficoll-Paque PLUS (GE Healthcare Bio-Sciences AB, 17-1440-02). $CD4^+$ T cells were isolated from PBMCs using the Human CD4 subset Column Kit (R&D Systems, Minneapolis, MN, USA). Purified $CD4^+$ T cells (1×10^6/well) were cultured in complete RPMI medium 1640 in 24-well plates at 37℃ in 5% CO_2 in the presence or absence of *P. citri* extract (50 μg/well) and with irradiated PBMCs as a source of antigen-presenting cells (APCs; 1×10^6/well). Stimulated $CD4^+$ T cells were harvested for *Tslp* and *Il-4* gene expression analysis by real-time PCR (9).

TSLP-specific antibody blocking experiment

The role of TSLP was assessed by culturing PBMCs (1×10^6 cells/well) in the presence of *P. citri* extract (50 μg/well) and an anti-human-TSLP antibody (1 μg/well; R&D Systems, Inc., Minneapolis, MN, USA). This monoclonal antibody can neutralize the bioactivity of TSLP *in vitro*. Cells were harvested for analysis of *Tslp* and *Il-4* gene expression by real-time PCR, and supernatants were collected and tested for the presence of TSLP, IL-4, and IgE.

Tslp and Il-4 gene expression as assessed by real-time PCR

Quantitative real-time RT-PCR was performed on a Bio-Rad system. Hypoxanthine-guanine phosphoribosyltransferase was used as a reference for sample normalization (Superarray Bioscience, Frederick, MD, USA). Total RNAs were prepared from PBMCs and $CD4^+$ T cells using an RNeasy Plus Mini Kit (QIAGEN, Cat. No. 74134), and cDNA was produced using a Reaction Ready First Strand cDNA Synthesis Kit (Superarray Bioscience). *Tslp* and *Il-4* mRNA were quantified using a real-time PCR system utilizing RT^2 primers (Superarray Bioscience). The relative quantitative real-time PCR data were calculated based on the $2^{-\Delta\Delta Ct}$ method (10).

TSLP, IL-4, and total IgE detection by ELISA

Supernatants were collected and tested for the presence of TSLP, IL-4, and total IgE. Quantitation of these cytokines was performed with the aid of a DuoSet ELISA Development System (R&D Systems, Inc.).

RESULTS

P. citri can induce the production of IgE and TSLP

TSLP plays a role in protease allergen-induced T_H2 inflammatory responses. Der p1 is a house dust mite allergen that is known to induce the T_H2 immune response (11). *P.*

citri (citrus red mite) infects, among others, tangerine trees on Jeju Island South Korea, and is also a known allergen that can induce T_H2 immune responses (5). However, the mechanism underlying the *P. citri*-induced T_H2 inflammatory responses are not yet known. Sokol *et al*. showed that the protease allergen causes the release of TSLP-induced IgE from basophils (2).

We studied the production of TSLP in *P. citri*-induced T_H2 inflammatory responses. First, we assessed IgE, which is released in response to the activation of allergen-specific T_H2 cells by B cells (1). The *P. citri* extract significantly affected the total IgE concentration, with levels increasing

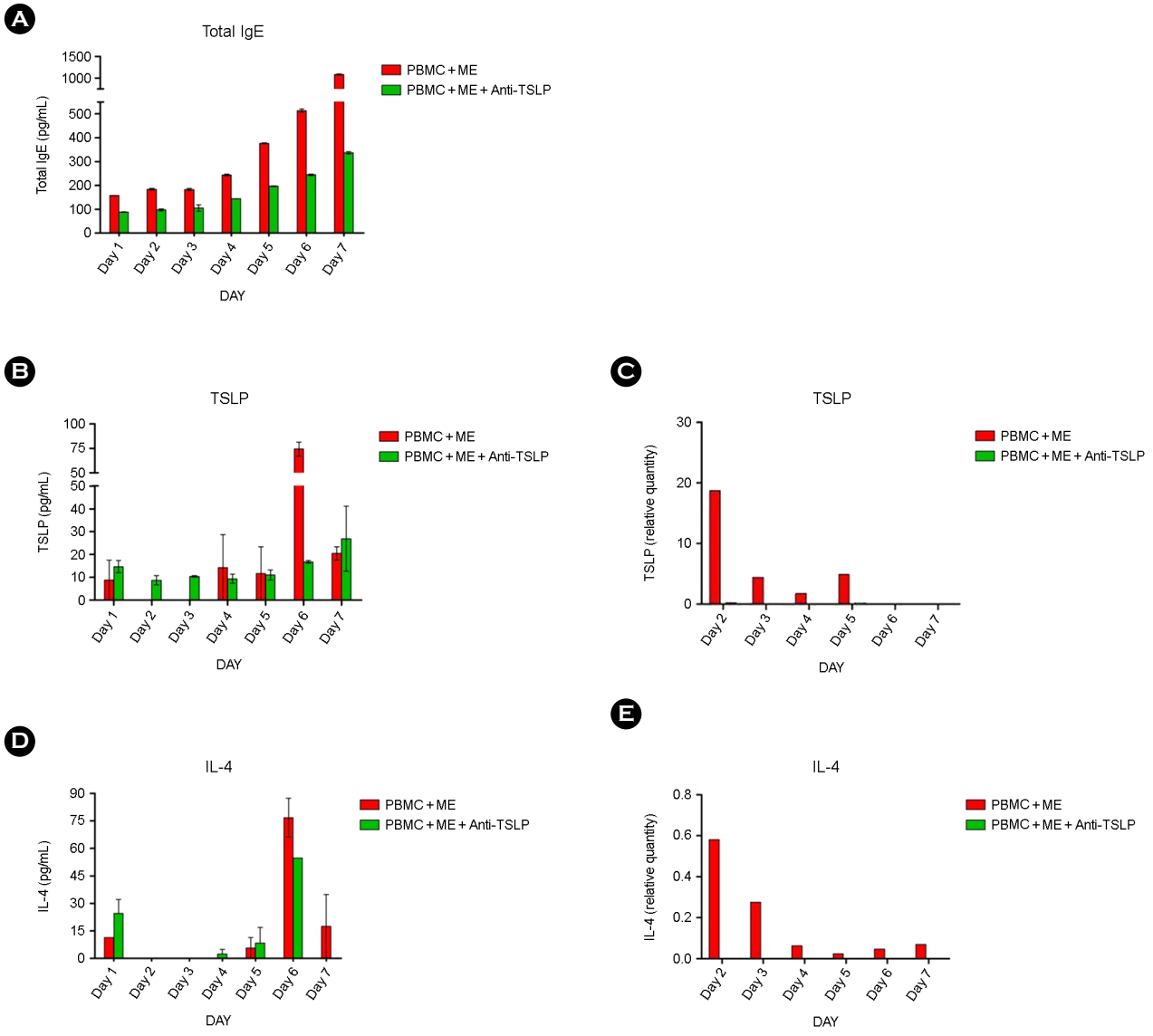

Figure 1. *P. citri* induces IgE secretion via the production of TSLP, and IL-4. IgE concentrations measured by ELISA (A). PBMCs were cultured with *P. citri* extract (50 μg/well). The role of TSLP in IgE secretion was assessed by culturing PBMCs with *P. citri* extract (50 μg/well) and anti-human-TSLP antibody (1 μg/well). TSLP production (B) and *Tslp* gene expression (C) were measured by ELISA and real-time PCR, respectively. IL-4 production (D) and *Il4* gene expression (E) were measured by ELISA and real-time PCR, respectively. ELISA, Enzyme-linked immunosorbent assay; PBMCs, human peripheral blood mononuclear cells; ME, *P. citri* extracts; Anti-TSLP: anti-human TSLP antibody.

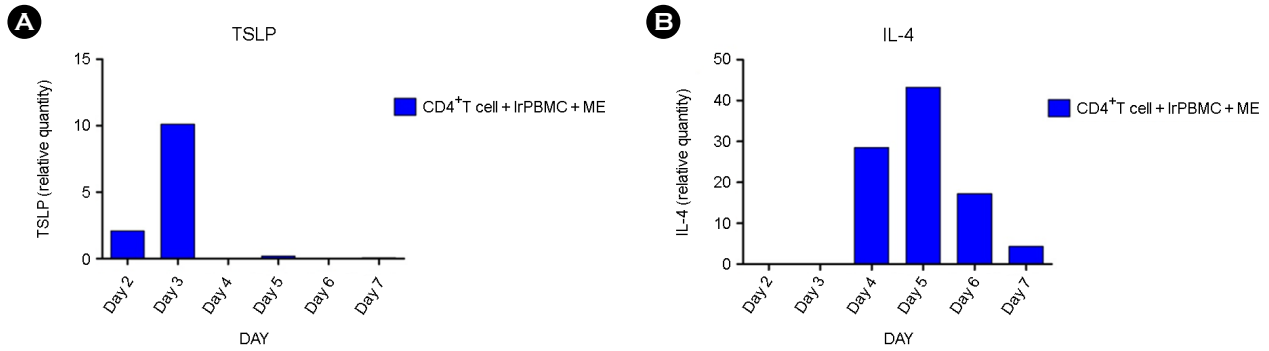

Figure 2. TSLP and IL-4 were produced by human CD4$^+$T cells following stimulation with *P. citri* extract. Real-time PCR analysis of *Tslp* (A) and *Il4* (B) gene expression in human CD4$^+$ T cells that had been incubated with *P. citri* extract (50 μg/well). Irradiated PBMCs were used as a source of APCs and purified CD4$^+$ T cells. IrPBMC, Irradiated human peripheral blood mononuclear cells; ME, *P. citri* extracts.

exponentially from day 2 to day 7 of culture. In addition, production of IgE by *P. citri*-extract-stimulated PBMCs reached a maximum after 7 days of culture (Fig. 1A).

TSLP secretion by PBMCs was also dramatically increased following 6 days of culture with *P. citri* extract (Fig. 1B). The mRNA relative expression of *Tslp* were also noticeable from day 2 to day 5, as shown in Fig. 1C. The expression of *Tslp* increased from day 2 to day 3 when human CD4$^+$ T cells were cultured with *P. citri* extract and irradiated PBMCs as a source of APCs (Fig. 2A).

P. citri-induced-TSLP can induce the production of IL-4

IL-4 plays a critical role in priming naïve CD4$^+$ T cells to become T_H2 cells (12, 13), and contributes to isotype switching between IgG_1 and IgE, and is thus an important factor associated with allergy and asthma (1).

To evaluate the production or expression of TSLP and IL-4, and *Tslp* and *Il4*, respectively, we determined the concentrations of TSLP and IL-4, and gene expressions of *Tslp*, *Il-4* in PBMCs stimulated with *P. citri* extract and human CD4$^+$ T cells and in irradiated PBMCs stimulated with *P. citri* extract. Concentrations of IL-4 reached a maximum on day 6 of culture when PBMCs were incubated with *P. citri* (Fig. 1D). The relative mRNA relative expression of *Il-4* in human CD4$^+$ T cells and irradiated PBMCs incubated with *P. citri* extract after *Tslp* gene expression increased from day 4 to day 5, and then decreased from day 6 to day 7 (Fig. 2B). This suggests that *P. citri*-induced-TSLP causes the production of IL-4 in *P. citri*-mediated-T_H2 allergic disease.

The role of TSLP in *P. citri*-mediated IgE secretion and IL4 production

To investigate the role of TSLP in total IgE secretion and IL-4 production, we incubated *P. citri* extract-stimulated PBMCs with anti-human-TSLP antibody. We found that incubation with the anti-TSLP antibody significantly reduced total IgE secretion from day 1 to day 7 (Fig. 1A); IL-4 production was also reduced (Fig. 1D), and no *Il-4* mRNA was detected (Fig. 1E). Thus, *P. citri*-induced TSLP plays an important role in IgE and IL-4 production.

DISCUSSION

P. citri infects citrus trees and is known to be a potent allergen associated with occupational allergy in humans (5). However, the mechanism underlying the *P. citri*-mediated T_H2 immune response is still not yet completely understood.

In this study, we reveal the mechanism underlying *P. citri*-induced IgE production, and show that PBMCs respond to stimulation with *P. citri* extract by releasing IgE, TSLP, and IL-4.

We found that *Tslp* and *Il4* are expressed by CD4$^+$ T

cells in response to *P. citri* extract and that the secretion of IgE and IL-4 is greatly reduced when PBMCs are incubated with a monoclonal antibody raised against human TSLP. Neutralization of TSLP resulted in inhibition of *P. citri*-induced IgE secretion, which suggests that production of this cytokine in response to *P. citri* is critical in the T_H2 immune response.

Many studies have shown that basophils-derived TSLP and IL-4 has been linked to T_H2 differentiation (2, 6, 8, 13~15). Sokol *et al*. showed that TSLP is produced by basophils and plays an important role in the initiation of T_H2 differentiation (2), and Seder *et al*. showed that basophils can produce large amounts of IL-4 (16). However, Omori *et al.* showed that TSLP can directly prime T_H2 differentiation without APCs, and TSLP-treated naïve $CD4^+$ T cells are potentially capable of producing IL-4 (8).

Our results did not reveal the source of the produced IL-4, which could be either basophils or $CD4^+$ T cells. However, we suggest that both TSLP and IL-4 can drive the T_H2 immune response in two ways: through the activation of B cells or by activating naïve $CD4^+$ T cells via basophils (Fig. 1B, 1C, 1D, 1E, 2A, and 2B). Further study is required regarding the source of the IL-4 released following in *P. citri*-induced IgE production (i.e., basophils, $CD4^+$ T cells, or both).

In summary, we show that *P. citri* is capable of directly driving the T_H2 immune response through the production of TSLP and IL-4, and for the first time show that the production of TSLP in response to *P. citri* plays a critical role in occupational asthma (i.e., IgE-mediated broncho-constriction).

REFERENCES

1) Kindt TJ, Goldsby RA, Osborne RA. Kuby Immunology 6th edition. Freeman, 2007;pp 307:384-5.
2) Sokol CL, Barton GM, Farr AG, Medzhitov R. A mechanism for the initiation of allergen-induced T helper type 2 responses. Nat Immunol 2008;9:310-8.
3) Min B, Paul WE. Basophils: in the spotlight at last. Nat Immunol 2008;9:223-5.
4) Finkelman FD, Urban JF Jr. Cytokines: making the right choice. Parasitol Today 1992;8:311-4.
5) Kim YK, Son JW, Kim HY, Park HS, Lee MH, Cho SH, *et al*. New occupational allergen in citrus farmers: citrus red mite (*Panonychus citri*). Ann Allergy Asthma Immunol 1999;82:223-8.
6) Liu YJ. Thymic stromal lymphopoietin: master switch for allergic inflammation. J Exp Med 2006;203:269-73.
7) Liu YJ, Soumelis V, Watanabe N, Ito T, Wang YH, Malefyt Rde W, *et al*. TSLP: an epithelial cell cytokine that regulates T cell differentiation by conditioning dendritic cell maturation. Annu Rev Immunol 2007;25:193-219.
8) Omori M, Ziegler S. Induction of IL-4 expression in CD4(+) T cells by thymic stromal lymphopoietin. J Immunol 2007;178:1396-404.
9) Hong J, Li N, Zhang X, Zheng B, Zhang JZ. Induction of $CD4^+CD25^+$regulatory T cells by copolymer-I through activation of transcription factor Foxp3. Proc Natl Acad Sci U S A 2005;102:6449-54.
10) Livak KH, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 2001;25:402-8.
11) Ghaemmaghami AM, Gough L, Sewell HF, Shakib F. The proteolytic activity of the major dust mite allergen Derp 1 conditions dendritic cells to produce less interleukin-12: allergen-induced Th2 bias determined at the dendritic cell level. Clin Exp Allergy 2002;32:1468-75.
12) Le Gros G, Ben-Sasson SZ, Seder R, Finkelman FD, Paul WE. Generation of interleukin 4 (IL-4)-producing cells *in vivo* and *in vitro*: IL-2 and IL-4 are required for in vitro generation of IL-4-producing cells. J Exp Med 1990;172:921-9.
13) Swain SL, Weinberg AD, English M, Huston G. IL-4 directs the development of Th2-like helper effectors. J Immunol 1990;145:3796-806.
14) Soumelis V, Reche PA, Kanzler H, Yuan W, Edward G, Homey B, *et al*. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. Nat Immunol 2002;3:673-80.
15) Gilliet M, Soumelis V, Watanabe N, Hanabuchi S,

Antonenko S, de Waal-Malefyt R, *et al*. Human dendritic cells activated by TSLP and CD40L induce proallergic cytotoxic T cells. J Exp Med 2003;197:1059-63.

16) Seder RA, Paul WE, Dvorak AM, Sharkis SJ, Kagey-Sobotka A, Niv Y, *et al*. Mouse splenic and bone marrow cell populations that express high-affinity Fc epsilon receptors and produce interleukin 4 are highly enriched in basophils. Proc Natl Acad Sci U S A 1991; 88:2835-9.