Clinics in Oncology

Research Article
Published: 10 Oct, 2017

Effects of Vialinins A and B on Murine Splenocytes Sensitized with Ovalbumin

*Jun-ichi Onose, Fumiyo Sekiya, Aki Shiomitsu, Yasukiyo Yoshioka, Kouichi Sugaya and Naoki Abe**

Department of Nutritional Science and Food Safety, Faculty of Applied Bio-science, Tokyo University of Agriculture, Japan

Abstract

Vialinins A and B, as strong inhibitors of the production and release of tumor necrosis factor (TNF)-α, have been isolated from the edible Chinese mush room, *Thelephoravialis*. Here we investigated the inhibitory effects of vialinins A and B on the immune system. Splenocytes obtained from ovalbumin (OVA)-sensitized BALB/c mice were challenged with OVA in the presence of vialinins A and B, and cytokine levels in the medium of cultured cells were measured. Vialinins A and B inhibited production of OVA-specific immunoglobulin (Ig)E and Th2-type cytokines (interleukin (IL)-4, IL-5, and IL-10) but not production ofTh1-type cytokines (interferon-γ, IL-2, and IL-12). Flow cytometric assay showed a significantly higher percentage of regulatory T cells (CD25- and Foxp3-positive T cells) among splenocytes cultured with OVA and vialinins A and B than among those cultured with OVA alone. This offers a first demonstration that vialinins A and B inhibit antigen-specific IgE- and Th2-type cytokines and regulation of regulatory T cells, suggesting the utility of vialinins A and B in preventing deleterious immune responses.

Introduction

Mushrooms have been used for centuries as folk medicines and food. The functionality of mush rooms in disease prevention and medicinal treatment has been passed down through generations. Many recent studies have shown significant agreement between the traditional uses of fungi in the treatment of specific symptoms and experimental anti-bacterial, anti-fungal, anti-cancer, and anti-viral activities in laboratory trials [1]. Furthermore, medicinal mush rooms are reportedly effective against inflammation [2]. Screening of medicinal mushrooms for bioactivity is thus extremely important to identify sources of potential therapeutic agents. Vialinins A and B were isolated from the dried fruiting bodies of *Thelephoravialis* (Thelephoraceae family), a Chinese mushroom popular for its special flavor and taste. Moreover, this mushroom has long been used for the treatment of low back pain and limb paralysis in China. Previous studies have revealed that vialin in A displays a powerful 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical-scavenging activity [3], and inhibits the antigen-induced production and release of tumor necrosis factor (TNF)-α from rat basophilic leukemia (RBL-2H3) cells (IC_{50}:a half maximal inhibitory concentration = 0.09 nM) and from murine bone marrow-derived mast cells (IC_{50} = 0.04 nM), compared with the clinical immunosuppressant tacrolimus used in the same run as a positive standard (IC_{50}= 0.25 nM) [4,5]. Vialinin B strongly inhibited TNF-α release induced by antigen from RBL-2H3 cells (IC_{50} = 0.02 nM) [6].On the other hand, analogs of vialinins A and B, comprisingganbajuninsB, D, and E, atromentin, and cycloleucomelone, exhibited no inhibitory activity against TNF-α production in RBL-2H3 cells [4,6].

Host immune responses are characterized by T-cell activation in response to antigen stimulation, leading to the differentiation of effector T-cell subtypes that, in turn, are characterized by distinct cytokine secretion and enzymatic profiles leading to specific effector functions. Efficient host defense against invading pathogenic microorganisms is achieved through coordination of complex signaling networks that link the innate and adaptive immune systems. Upon interaction with cognate antigen presented by antigen-presenting cells such as dendritic cells (DCs), CD4+ T cells can differentiate into a variety of effector subsets, Th1 cells, Th2 cells, Th17 cells, and induced regulatory T cells(iTregs). The differentiation decision is governed predominantly by the cytokines present in the microenvironment, and, to some extent, by the strength of the interaction of the T-cell antigen receptor with antigen [7,8]. Th1 responses are induced by IL-12, composed of IL-12α and IL-12β, triggering interferon (IFN)-γ production [9]. Th2 responses are characterized by

OPEN ACCESS

Correspondence:
Naoki Abe, Department of Nutritional Science and Food Safety, Faculty of Applied Bio-science, Tokyo University of Agriculture, Japan, Tel:+81-3-5477-2450; Fax: +81-3-5477-2735; E-mail: j1onose@nodai.ac.jp

Received Date: *26 Aug 2017*
Accepted Date: *20 Sep 2017*
Published Date: *10 Oct 2017*

Citation:
Jun-ichi Onose, Sekiya F, Shiomitsu A, Yoshioka Y, Sugaya K, Abe N. Effects of Vialinins A and B on Murine Splenocytes Sensitized with Ovalbumin. Clin Oncol. 2017; 2: 1357.

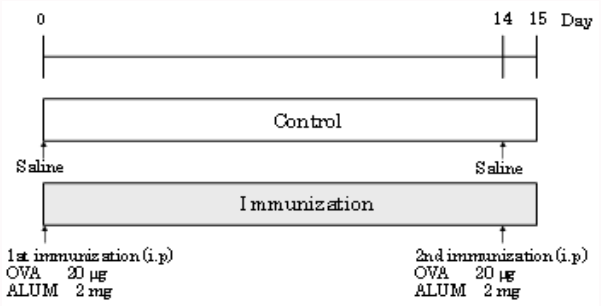

Figure 1: Experimental design and immunization.

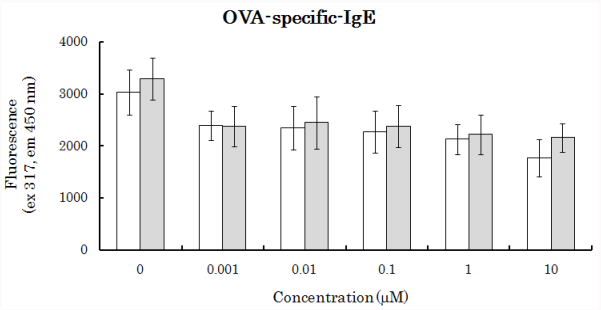

Figure 2: Effects of vialinins A and B on OVA-specific IgE production in murine splenocytes. White bar,treated with vialinin A; grey bar,treated with vialinin B. Data expressed as mean ± S.D. of five independent experiments. Significance of differences from control values was estimated using Student's t-test (**p < 0.01, *p< 0.05).

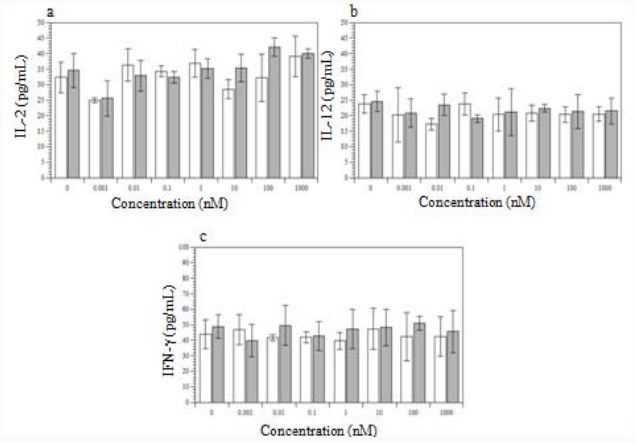

Figure 3: Effects of vialinins A and B on Th1-type cytokines production in murine splenocytes. White bar, treated with vialinin A; grey bar, treated with vialinin B. Data expressed as mean ± S.D. of five independent experiments.

IL-4 and IL-13 production [10]. In addition, Th17 cells, characterized by IL-17 production, cause recrudescence of autoimmune disease [11]. Uncontrolled Th1 responses can lead to necrosis and tissue damage, whereas exaggerated responses by Th2 cells can induce asthma and allergy, and can also lead to tissue inflammation and fibrosis [12]. Furthermore, transcription factors play important roles in many immune disorders. Th1-cell differentiation requires the action of T-box transcription factor (T-bet), Th2 differentiation requires the action of GATA-binding protein 3 (GATA3), Th17-cell differentiation requires retinoid-related orphan receptor (ROR) γt, and Treg differentiation requires forkhead box P3 (Foxp3). The present study examined the effects of vialinins A and B on the

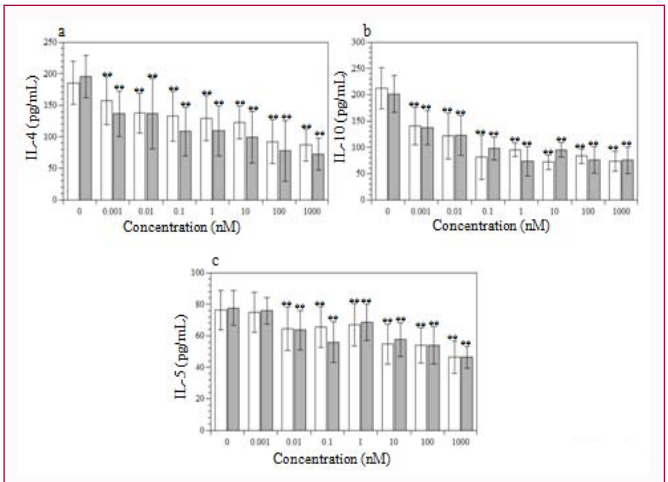

Figure 4: Effects of vialinins A and B on Th1-type cytokines production in murine splenocytes. White bar, treated with vialinin A; grey bar, treated withvialinin B. Data expressed as mean ± S.D. of five independent experiments.Significance of differences from control values was estimated using Student's t-test (**$p < 0.01$).

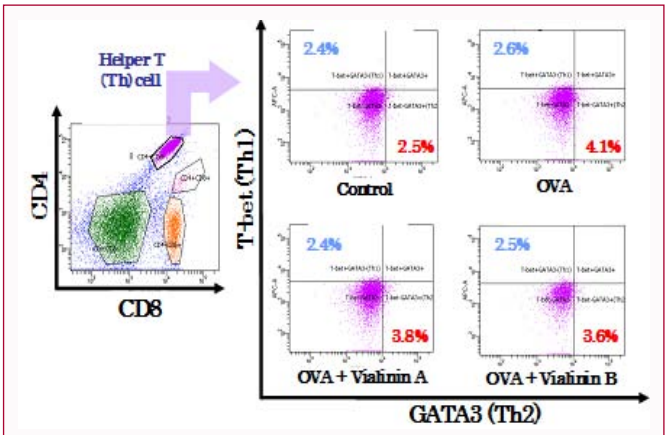

Figure 5: Expression of T-bet and GATA3 on murine splenocytes. Splenocytes were isolated from the control, OVA treatment, OVA + vialinin A and OVA + vialinin B groups. Histograms are representative of five independent experiments.

production of specific immunoglobulin (Ig)E antibody in murine splenocytes sensitized with ovalbumin (OVA).Furthermore, to clarify the mechanisms underlying the inhibition of specific IgE production, we examined the pattern of cytokine production by vialinin-stimulated splenocytes from murine splenocytes sensitized with OVA. In addition, we investigated the proportions of helper T cells, Th1 cells, Th2 cells, Th17 cells, and Tregs in vialinin-stimulated splenocytes from murine splenocytes sensitized with OVA.

Materials and Methods

Animals

Inbred specific-pathogen-free BALB/c mice (male, 6weeks old) were purchased from Charles River Japan (Yokohama, Japan). Mice were maintained in atemperature- and light-controlled environment with free access to sterile diet and water, and were acclimatized for at least 1 week before the start of the study.All experiments were conducted according to the *Guide for the Care and Use of Laboratory Animals from theJapan Neuroscience Society* and the *Guide for the Tokyo University of Agriculture.*

Five mice were sensitized by intraperitoneal injection of 20 μg of

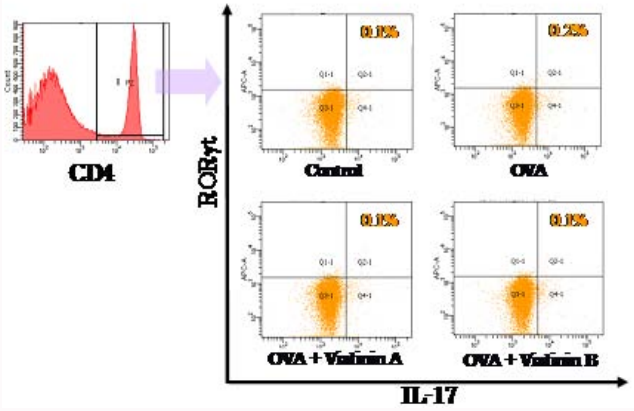

Figure 6: Expression of RORt and IL-17 on murine splenocytes. Splenocytes were isolated from the control, OVA treatment, OVA + vialinin A and OVA + vialinin B groups. Histograms are representative of five independent experiments.

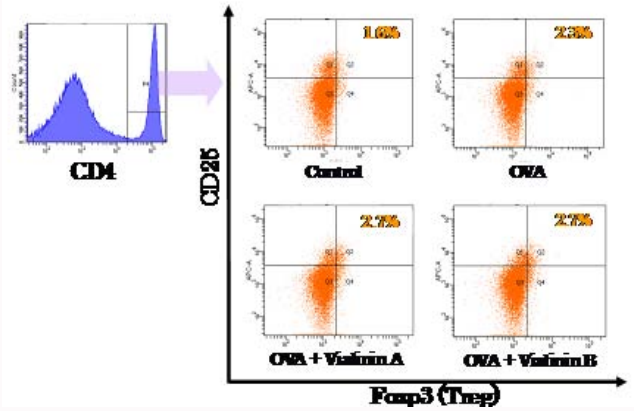

Figure 7: Expression of CD25 and Foxp3 on murine splenocytes. Splenocytes were isolated from the control, OVA treatment, OVA + vialinin A and OVA + vialinin B groups. Histograms are representative of five independent experiments.

OVA and 2 mg of aluminum hydrate adjuvant (ALUM ($Al(OH)_3$); LSL, Shiga, Japan)in a total volume of 400 µl. Two weeks after the first injection, they were given a booster injection of the same doses of the antigens. The next day the mice were humanely sacrificed, and their spleens were harvested. The immunization schedule is shown in Figure 1.

Preparation and stimulation of murine splenocytes *in vitro*

Mice were sacrificed by cervical dislocation, and after removing their spleens aseptically, cell suspensions were prepared by passing the spleens through a sterile cells trainer (FALCON 35-2350). The cell suspensions we rewashed twice in RPMI 1640 medium supplemented with10% FBS, 2 mML-glutamine, 1 mM sodium pyruvate,50 U/ml kanamycin sulfate, and 25 nM 2-ME, and they were adjusted to a density of 5 $\times 10^6$ cells/ml. Cells were plated at 2 ml/well in 24-well cell culture clusters(Corning, NY) and challenged with OVA at a final concentration of 100 µg/ml. Vailinins A or B (final concentration:1 µM), or saline was added to the 24-wellculture clusters, and it was incubated at 37 ˚C in a CO_2 incubator for 3-14 days. Vialinins A and B were used as synthetic compounds [13,14]. Supernatants and cells were then harvested to measure cytokine production and cell surface markers.

OVA specific-IgE and cytokine production *in vitro*

The mouse serum OVA specific-IgE was determined by the method. with some modifications. The cytokines examined in this study were IFN-γ, IL-2, IL-4, IL-5, IL-10, and IL-12. The amounts of these cytokines in the culture medium after incubation were measured with ELISA kits (R&D Systems, Minneapolis, MN)based on the quantitative sandwich enzyme immunoassay technique. Absorbance was measured at 450nm using a precision micro plate reader.

Flow cytometric analysis

Flow cytometric analysis was performed using a FACSAria flow cytometer (BD Biosciences, San Jose, CA, USA)equipped with a 488 nm argon laser and detectors for forward scatter (FSC) and 90°light scatter (side scatter, SSC) and for FL1 (band pass filter wavelength, 530 nm) and FL2 (585 nm) fluorescence emission in the green part and red/orange part, respectively, of the spectrum. Splenocytes were stained with phycoerythrin(PE)-labeled Cy7 Rat anti-mouse CD4, allophycocyanin (APC)-labeled Cy7 Rat anti-mouse CD8a, Alexa Fluor 488 Rat anti-mouse GATA3, PE-labeled Rat anti-mouse IL-17a, APC-labeled Rat ant-mouse CD25, Alexa Fluor 488 Rat anti-mouse Foxp3 (BD Biosciences), Alexa Fluor 647 Rat anti-mouse T-bet (Santa Cruz Biotechnology, Dallas, Texas, USA), Anti-human/mouse RORγt/RORC2/NR1F3-APC (R&D Systems). Fluorescence overlap was compensated electronically by using splenocytes stained with single colors, and then 10,000 cells were acquired and stored for each analysis. Splenocytes were identified by their characteristic appearance on a dot plot of FSC versus SSC and electronically gated to exclude platelets, red cells, and dead-cell debris. The gate was the same for splenocytes co-cultured with OVA and vialinin A, with OVA and vialinin B, and with OVA alone. The results are reported as percentages of positive cells within a gate. The absolute number of each type of cell was calculated from the percentage of each type of positive cell and the total number of splenocytes.

Statistical analysis

Numerical data are expressed as mean ± standard deviation of the mean. Differences were evaluated using Student's *t*-test, and values of $p < 0.01$ were considered statistically significant.

Results

Effects of vialinins A and B on production of OVA-specific IgE from splenocytes

We investigated whether vialinins A and B suppress OVA-specific IgE production. Although control splenocytes produced OVA-specific IgE, splenocytes treated with vialinins A and B appeared to suppress production of OVA-specific IgEin low concentration (Figure. 2).

Effects of vialinins A and B on production of cytokine from splenocytes

To clarify the inhibitory mechanisms of OVA-specific IgE production in mice, we investigated production of Th1- and Th2-type cytokines from murine splenocytes stimulated with OVA *in vitro*. Cytokine release was expressed in the form of activity released into the medium as a percentage of total activity. Vialinins A and B did not influence Th1-type cytokine IL-12, IL-2 orIFN-γ (Figure.3). However vialinins A and B also inhibited the Th2-type cytokines IL-4, IL-5, and IL-10 in a dose-dependent manner (Figure.4).

Flow cytometricanalysis of splenocytes

To investigate the effects of vialinins A and B on differentiation of murine splenocytes treated with OVA, we undertook phenotypic analysis of murine splenocytes using FCM. Addition of vialinin A or B to OVA-sensitive murine splenocytes decreased GATA3-positive T cells,Th2 cells, but did not influence T-bet-positive T cells, Th1 cells (Figure 5).We also investigated the effects of vialinins A and B on differentiation ofTh17 and Treg cells. Differentiation of Th17 cells, identified as RORγT-positive T cells, was not influenced by the presence of vialinins A and B (Figure 6). However, Tregs, identified as Foxp3-positive T cells, were increased among OVA-sensitive murine splenocytes treated with vialinins A and B (Figure 7).

Discussion

We first demonstrated that the effects of vialinins A and B on OVA-specific IgE production by urine splenocytes. Vialinins A and B inhibited OVA-specific IgE production at concentrations of 0.001μM each (Figure 2). It has reported that oligodeoxynucleotide from Bifidobacteriumlongum inhibited OVA-specific IgE production by murine splenocytes [15]. Vialinins were not strongly inhibited as compared with oligodeoxynucleotide but there were significantly inhibited. Next, we investigated the effects of vialinins A and B on Th1- and Th2-type cytokine production by murine splenocytes, because IgE production is related to humoral immune response. CD4+ helper T cells are subpopulations of two cell types, Th1 and Th2, defined based on the different patterns of cytokine production [16,17]. The balance of these two types of cells is considered to be important for maintaining homeostasis in the host. Once this balance becomes disturbed, various immunological diseases, such as allergies and intestinal inflammation, can occur due to circumvention of the host defense mechanisms. Regulation of these two types of cell seems important for preserving host immune response, including IgE and cytokine production. Vialinins A and B inhibited the Th2-type cytokines IL-4, IL-5, and IL-10 from murine splenocytes treated with OVA in a dose-dependent manner (Figure 4), but did not influence theTh1-type cytokines IL-2, IL-12, and IFN-γ (Figure 3). We therefore next investigated the effects of vialinins A and B on differentiation of murine splenocytes, particularly helper T cells. Vialinins A and B suppressed differentiation of GATA3-positive cells, representing Th2 cells, induced by OVA but showed no influence on the differentiation of Th1 cells, as T-bet-positive cells (Figure 5). These findings suggest that vialinins inhibit certain cytokines but do not influence the balance of helper T cells (the Th1/Th2 paradigm). Recently, Th1/Th2/Th17 and regulatory T-cell paradigm was spread into the mainstream of mutually interacting immune network [18]. Vialinins A and B did not influence differentiation of RORγt-positive Th17 cells (Figure 6), but increased that of Tregs, as CD25- and Foxp3-positive T cells, in murine splenocytes treated with OVA (Figure 7).

One of the deubiquitinating enzyme (DUB):ubiquitin-specific protease 4 (USP4), reportedly promotes Th17 cell differentiation in human naive T cells [19]. We have identified ubiquitin-specific peptidase 5 (USP5), as a target molecule of vialinin A in RBL-2H3 cells, and vialinin A inhibited USP5enzymatic activity *in vitro*. In addition, vialinin A also strongly inhibited the activity of USP4 [20]. Though TNF-α production was decreased in USP5 siRNA-knockdown RBL-2H3 cells, no relationship was identified between USP4 and TNF-α release [21]. The observation that vialinins as potent USP4 inhibitors gave no differentiation activities of RORγt-positive Th17 in this OVA treatment study, suggests that functions of DUBs may be different depending on the sensitization level of T-cells.

Regulatory CD4+CD25+ T cells (Tregs) mediating immune balance and immunopathologicalcontrol in the thymus are called natural Tregs (nTregs) [12]. These nTregscan reach the periphery as functional suppressor cells [22]. Tregs exert suppressive functions either directly by producing IL-10 and TGF-β, or indirectly via dendritic cells [23]. In some settings, a complex network of both Foxp3+ Tregs and combinations of IL-10 and TGF-β has been found to play an important role in controlling host immune responses. However, reversal of these regulatory settings has been shown to result in parasite clearance [24]. These findings suggest that vialinins might control the immune balance via inhibition of Th2-type cytokines and regulation of nTregsaccording to the Th1/Th2/Th17 and regulatory T-cell paradigm. Increasing Tregsis reportedly related to small cell lung cancer and late-stage ovarian cancer [25], and immune tolerance in the fetus and womb [26]. Tregs, as both nTregs and iTregs, constitute an indispensable component of the immune system. Further elucidation of the cellular functions of Tregs and the molecular function of vialinins will contribute to our understanding of immune tolerance and homeostasis and provide insights into methods for achieving better control of immune responses for the benefit of the host. Vialinins A and B are semi-specific DUB inhibitors and therefore they have multiple points of action. The ubiquitin pathway is necessary at all stages of development in eukaryotic cells. Dynamic modification of a substrate protein by ubiquitin can modify functions, localizations, and fate in the cell [27]. Ubiquitin conjugation depends on a cascade of enzymes, and its removal is mediated by DUBs. In recent years, attention has been focused on the function of the ubiquitin-proteasome system, but many parts remain unknown. Understanding the function of the ubiquitin-proteasome system in immunology has contributed to the understanding of immune-related diseases [28-30]. In conclusion, we demonstrated that vialinins A and B inhibited Th2-type cytokines and antigen-specific IgE production from murine splenocytes. Vialinins A and B also controlled nTregs. Vialinins may be beneficial in preventing various types of deleterious immune response.

References

1. Fabricant DS, Farnsworth NR. The value of plants used in traditional medicine for drug discovery. Environ Health Perspect. 2001;109:69-75.
2. Jeong JW, Lee HH, Han MH, Kim GY, Hong SH, Park C, Choi YH. Ethanol extract of Poriacocos reduces the production of inflammatory mediators by suppressing the NF-kappaB signaling pathway in lipopolysaccharide-stimulated RAW 264.7 macrophages. BMC Complement Altern Med. 2014;14:101.
3. Xie C, Koshino H, Esumi Y, Takahashi S, Yoshikawa K, Abe N, et al. a novel 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenger from an edible mushroom in China. BiosciBiotechnolBiochem.2005;69:2326.
4. Onose J, Xie C, Ye Y, Sugaya Q, Takahashi K, Koshino S, et al. Vitamin A, a novel potent inhibitor of TNF - alpha production from RBL-2H3 cells. Pharm Bull. 2008;31:831.
5. Onose J, Yoshioka Y, Ye Y, SugayaQ, Yajima K, Taniguch iA, et al. Inhibitory effects of Vitamin A and its analog on tumor necrosis factor - α release and production from RBL-2H3 cells. Cell Immunol. 2012;279:140.
6. Xie C, Koshino H, Esumi Y, Onose J, Yoshikawa K, Abe N. Vialinin B, a novel potent inhibitor of TNF-alpha production, isolated from an edible mushroom, Thelephoravialis. Bioorg Med Chem Lett. 2006;16(20):5424-6.
7. Boyton RJ, Altmann DM. Is selection for TCR affinity a factor in cytokine polarization? Trends Immunol. 2002;23(11):526-9.

8. Zhou L, Chong MM, Littman DR. Plasticity of CD4+ T cell lineage differentiation. Immunity. 2009;30(5):646-55.

9. Dalton DK, Pitts-Meek S, Keshav S, Figari IS, Bradley A, Stewart TA. Multiple defects of immune-cell function in mice with disrupted interferon-γ genes. Science. 1993;259(5102):1739-42.

10. Goerdt S, Orfanos CE. Other functions, other genes: alternative activation of antigen-presenting cells. Immunity. 1999;10(2):137-42.

11. Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD, et al. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. J Exp Med. 2005;201:233-40.

12. Dewals B, Hoving JC, Horsnell WG, Brombacher F. Control of Schistosomamansoni egg-induced inflammation by IL-4-responsive CD4+ CD25-CD103+ Foxp3-cells is IL-10-dependent. Eur J Immunol. 2010;40(10):2837-47.

13. Yue QY, Koshino H, Onose J, Yoshikawa K, Abe N, Takahashi S. First total synthesis of vialinin A, a novel and extremely potent inhibitor of TNF-alpha production.Org Lett. 2007;9(21):4131-4.

14. Ye YQ, Koshino H, Onose J, Yoshikawa K, Abe N, Takahashi S. Expeditious synthesis of vialinin B, an extremely potent inhibitor of TNF-alpha production.Org Lett. 2009;11(21):5074-7.

15. Takahashi N, Kitazawa H, Iwabuchi N, Xiao JZ, Miyaji K, Iwatsuki K, Saito T. Immunostimulatory oligodeoxynucleotide from Bifidobacteriumlongum suppresses Th2 immune responses in a murine model.Clin ExpImmunol. 2006;145(1):130-8.

16. Mosmann TR, Coffman RL. Heterogeneity of cytokine secretion patterns and functions of helper T cells. AdvImmunol. 1989;46:111-47.

17. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I Definition according to profiles of lymphokine activities and secreted proteins. J Immunol. 1986;175:5-14.

18. S Saito, A Nakashima, T Shima, M Ito. Th1/Th2/Th17 and Regulatory T-Cell Paradigm in Pregnancy. Am J ReprodImmunol. 2010;63(6):601-610.

19. Yang J, Xu P, Han L, Guo Z, Wang X, Chen Z, et al. Ubiquitin-specific protease 4 promotes Th17 cell function under inflammation by deubiquitinating and stabilizing RORγt. J Immunol. 2015;194(9):4094-7.

20. Okada K, Yue YQ, Taniguchi K, Yoshida A, Tomonori A. Vialinin A is a ubiquitin-specific peptidase inhibitor. Bioorg Med Chem Lett.2013;05:093.

21. Yoshioka Y, Ye YQ, Okada K, Taniguchi K, Yoshida A, Sugaya K, et al. Ubiquitin-specific peptidase 5, a target molecule of vialinin A, is a key molecule of TNF-α production in RBL-2H3 cells. PLoS One. 2013;8(12):e80931.

22. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor- chains (CD25): breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol. 1995;155:1151-64.

23. Belkaid Y, Tarbell K. Regulatory T cells in the control of host-microorganism interactions. Annu Rev Immunol. 2009,27:551-89.

24. Beiting DP, Gagliardo LF, Hesse M, Bliss SK, Meskill D, Appleton JA. Coordinated control of immunity to muscle stage Trichinellaspiralis by IL-10, regulatory T cells, and TGF-beta. J Immunol. 2007;178:1039-47.

25. Woo EY, Chu CS, Goletz TJ, Schlienger K, Yeh H, Coukos G, et al. Regulatory CD4+CD25+ T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. Cancer Res. 2001;61:4766-72.

26. Rowe JH, Ertelt JM, XinL. Pregnancy imprints regulatory memory that sustains anergy to fetal antigen. Nature. 2012;490:102-6.

27. Kerscher O, Felberbaum R, Hochstrasser M. Modification of proteins by ubiquitin and ubiquitin-like proteins. Annu Rev Cell Dev Biol. 2006;22:159-80.

28. Misaghi S, Balsara ZR, Catic A, Spooner E, Ploegh HL. Chlamydia trachomatis-derived deubiquitinating enzymes in mammalian cells during infection. MolMicrobiol.2006;61:142-50.

29. Kattenhorn LM, Korbel GA, Kessler BM, Spooner E, Ploegh HL. A deubiquitinating enzyme encoded by HSV-1 belongs to a family of cysteine proteases that is conserved across the family Herpesviridae. Mol Cell. 2005;19:547-57.

30. Angot A, Vergunst A, Genin S, PeetersN. Exploitation of eukaryotic ubiquitin signaling pathways by effectors translocated by bacterial type III and type IV secretion systems. PLoSPathog.2007;3:e3.