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Identification of anti-allergic effect of *Clonorchis sinensis*-derived protein venom allergen-like proteins (CsVAL)

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ABSTRACT

Previous studies demonstrated that *Clonochis sinensis*-derived crude antigens suppress development of allergic responses. We investigated the effects of *C. sinensis* venom allergen-like (CsVAL) proteins on immune-modulating activities in allergic inflammatory response. Using RBL-2H3 rat mast cells, we demonstrated that CsVAL inhibits antigen-induced β-hexosaminidase release from immunoglobulin E-sensitized RBL-2H3 cells, and this inhibitory activity occurs by suppressing Lyn phosphorylation and intracellular reactive oxygen species production. In addition, CsVAL peptide treatment inhibits activation of protein kinase C-α and extracellular signal-regulated kinase 1/2, which are involved in degranulation of immunoglobulin E-sensitized mast cells. Furthermore, immunization with CsVAL suppressed development of skin inflammation by assessing ear thickness and cutaneous infiltration by eosinophils and mast cells in oxazolone-induced contact hypersensitivity *in vivo* mouse model. These results suggest that CsVAL is a promising candidate as an effective mast cell inhibitor for allergic and inflammatory diseases.

1. Introduction

Recent studies have suggested that helminth infections or helminth-derived products could induce suppression of allergen-specific Th2 responses [1–3]. The inverse relationship between the allergic immune response and helminth infection has been described as the Hygiene Hypothesis, which postulates that a steady decline in exposure to viral, bacterial, and parasitic infection leads to an increase in allergic disorders [4]. Conditions such as asthma and atopic dermatitis are immune disorders caused by the hyperactivation of mast cells and overproduction of immunoglobulin E (IgE) in response to allergens [5,6]. The activation of mast cell by IgE is important to allergic reactions as well as host-protective immune responses against the helminthic parasites. Initial exposure to allergen induces the activation of Th2 cells and IgE synthesis, and subsequent exposure elicits widespread degranulation of IgE-sensitized mast cells and release of mediators such as histamine, thereby further aggravating the ongoing allergic process [5–7]. Because mast cell activation is initiated through the cross-linking of allergen-specific IgE bound to the high affinity receptors for IgE (FcεRI), FcεRI and IgE play a crucial role in the pathogenesis of Type I hypersensitivity reactions. Cross-linking of FcεRI with specific allergen strongly induces oxidative stress and activation of signaling pathways, leading to mast cell activation [8–10]. Production of high levels of intracellular reactive oxygen species (ROS) in mast cells induces histamine release from these cells, suggesting that ROS are necessary for the secretion of this mediator [11,12]. In addition, FcεRI cross-linking on mast cells activates early signaling cascades that leads to recruitment of the non-receptor tyrosine kinase, Lyn, to the receptor complex. Lyn activation induces maximum FcεRI aggregation through the phosphorylation of receptor subunits and simultaneous activation of signaling molecules such as phospholipase Cγ, which is important for activation of protein kinase C (PKC) isoforms required for optimal degranulation [8,10,13]. Lyn phosphorylation also leads to activation of mitogen-activated protein kinase (MAPK) pathways, including extracellular signal-regulated kinase (ERK)1/2 and p38 MAPK, that regulate the production of inflammatory cytokines [8,10,11].

The trematoda *Clonochis sinensis* is a liver fluke that dwells in the bile ducts of humans. *C. sinensis* infection is endemic predominantly in Asia, including Korea and China [3]. Because our previous study showed that *C. sinensis*-derived crude antigens suppress the development of allergic responses in animals [3], we hypothesized that *C. sinensis*-derived products may directly interfere with the allergen-specific Th2 response. Therefore, it was necessary to identify *C. sinensis*-derived antigens that exhibit a potent suppressive

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effect on allergic inflammation to understand the mechanisms related to the helminth-mediated anti-allergic effect. By predicting antigenic proteins based on the *C. sinensis* expressed sequence tags database, we identified and sequenced a multitude of antigen candidates that bound to sera from patients with *C. sinensis* infection. The anti-allergic effects of these antigenic proteins were determined using rat basophilic leukemia (RBL)-2H3 cells. One candidate, the *Clonochis sinensis* venom allergen-like proteins (CsVALs), significantly inhibited β-hexosaminidase release from RBL-2H3 cells. CsVAL proteins are members of a superfamily that possess a highly conserved α-β-α sperm-coating protein/Tpx-1/Ag5/PR-1/Sc7 (SCP/TAPS) structural domain, which is also present in *Schistosoma mansoni* (unpublished data). VAL proteins are expressed in many trematodes and nematodes, but their molecular functions remain unclear [14,15]. However, a recent study of SmVALs in *S. mansoni* suggested that these proteins may play critical roles as immunomodulators in the host–parasite interaction [16].

In this study, we used RBL-2H3 rat mast cells to examine the anti-allergic effects of the CsVAL peptide with regard to β–hexosaminidase release, intracellular ROS production, and activation of signaling pathways involved in mast cell degranulation and cytokine production. Furthermore, we investigated the prophylactic effect of immunization with CsVAL using a mouse model of oxazolone-induced contact hypersensitivity.

2. Materials and methods

2.1. Cell lines and animals

RBL-2H3 cells and human U266B1 multiple myeloma cells were obtained from the American Type Culture Collection (USA). Pathogen-free, female Balb/c mice aged 6 to 7 weeks were purchased from Orient Bio Inc. (Korea) and housed in a specific pathogen-free facility in individually ventilated and filtered cages. All animals used in this study were maintained and handled in strict accordance with institutional guidelines. The experiments were approved by the Committee on the Ethics of Animal Experiments of the Korean Centers for Disease Control and Prevention (Permit number KCDC-004-11-2A).

2.2. Reagents, antibodies, prediction and synthesis of epitope peptides

Dinitrophenyl-specific bovine serum albumin (DNP-BSA), anti-DNP-IgE, ketotifen fumarate, *p*-nitrophenyl *N*-acetyl-β-D-glucosamine, 2′-amino-3′-methoxyflavone (PD98059), *N*-acetyl-L-cysteine (NAC), lipopolysaccharide, 2,7-dichlorodihydrofluorerscein diacetate (DCFH-DA), and oxazolone were purchased from Sigma–Aldrich (USA). For analysis of changes in necrosis and apoptosis, FITC annexin V apoptosis kit I (BD Biosciences, USA) was used. For Western blot, anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-p38, and anti-p38 antibodies (Santa Cruz Biotechnology, USA), Anti-phospho-Lyn, anti-Lyn, anti-PKC-α and anti-phospho-PKC-α antibodies (Cell Signaling Technology. USA) were used. For flow cytometric analysis, splenocytes were stained with the following monoclonal antibodies: fluorescein isothiocyanate-conjugated anti-CD4 and phycoerythrin-conjugated anti-CD25 (eBioscience, USA). The BepiPred algorithm (www.cbs.dtu.dk/services/BepiPred) was used for the linear B-cell epitope prediction. The CsVAL peptide (GQNWAGGYDLQGAFNAWFDEYRNYNYANRS), CsAY15-1 (KQRHEE DDGFYTQGGSFYVSGKARRD-DGYG), CsAF09-1 (EEFKLKYKKTYSND DDELRFEIFKDNLLRA), and CsProR (DAPVPKSGGPDAPVP-KSGGPDA PVPKSGG) was synthesized by the Peptron Company (Korea).

2.3. Identification of antigenicity of synthetic peptides

C. sinensis-derived synthetic epitope peptides were coated in 96-well plate. The sera of patients infected with *C. sinensis* or healthy individual were diluted 1:300 respectively and incubated with coated peptides for 2 h at room temperature. Peroxidase-conjugated anti-human immunoglobulin G, as secondary antibody, was diluted 1:1000 and was applied to the wells. The absorbance was measured at a wavelength of 490 nm.

2.4. β-Hexosaminidase release assay

Cells were cultured in 48-well plates (2.5×10^6 cells/well) and sensitized with anti-DNP-IgE (500 ng/mL) for 16 h. To remove excess DNP-IgE before stimulation, sensitized RBL-2H3 cells were washed twice with Tyrodes' assay buffer (119 mM NaCl, 4.74 mM KCl, 2.5 mM $CaCl_2$, 1.19 mM $MgSO_4$, 10 mM HEPES, 5 mM glucose, 0.1% (w/v) BSA, pH 7.3). To stimulate the cells, 500 μg/mL DNP-BSA was added and cells were incubated at 37° for 1 h. Following stimulation, the supernatant was incubated with an equal volume of substrate solution (1 mM *p*-nitrophenyl *N*-acetyl-β-D-gulucosamine in 0.05 M citrate buffer, pH 4.5) at 37 °C for 1 h. As a control for total β-hexosaminidase concentration, the remaining cells were lysed and their supernatants were also incubated with an equal volume of substrate solution. The enzyme reaction was stopped by the addition of 0.05 M sodium bicarbonate buffer (pH 10.0) and the reaction product was measured as the absorbance at 450 nm using a microplate reader.

2.5. Intracellular ROS measurement

RBL-2H3 cells were sensitized with anti-DNP IgE overnight and incubated with 5 μM of DCFH-DA for 30 min. Cells were stimulated with DNP-BSA for 5 min. After stimulation, cells were immediately observed by fluorescence microscopy. To monitor real-time intracellular ROS production in live cells, the IncuCyte™ real-time imaging system (Essen Instruments Inc, USA) was used.

2.6. Establishment of the oxazolone-induced contact hypersensitivity (CHS) murine model

We established oxazolone-induced CHS in Balb/c mice as described [17]. Briefly, for sensitization, 25 μl oxazolone (100 mg/mL) in acetone/olive oil (4:1, v/v) was injected subcutaneously into the shaved flank skin of mice on day 0. Then, the animals were challenged with 10 μl 5% oxazolone on day 5 and with 2% oxazolone on the ears on days 6–14. Ear thickness was measured with a digimatic micrometer (Schering AG, Germany). For a prophylactic treatment with CsVAL peptide, Balb/c mice were immunized with CsVAL peptide (5 mg/kg) at the day 7 and day 1, respectively, before sensitization.

2.7. Histological analysis

Ear tissue was embedded in paraffin and cut into 6-μm thick sections for histological analysis. Tissue sections were stained with hematoxylin and eosin to demonstrate morphologic changes and eosinophil infiltration. To assess mast cell accumulation, the tissue sections were also stained with toluidine blue.

2.8. Statistical analysis

To determine the statistical significance of differences between the means of all variables was conducted using one-way analysis of variance (GraphPad Prism 3.0). Turkey's multiple-comparison test

was used for pairwise comparison of data from multiple groups. All comparisons with a P-value < 0.05 were considered statistically significant.

3. Results

3.1. CsVAL peptide inhibits antigen-induced β-hexosaminidase release in IgE-sensitized RBL-2H3 cells

To identify peptides that were capable to binding to the sera of patients infected with *C. sinensis*, we predicted antigenic determinants on a multitude of antigen candidates by using Bepipred, one of sequence-based predicts which are based on more advanced bioinformatical methods, and synthesized them to confirm their antigenic property. ELSIA assays showed that these predicted epitope peptides reacted with sera from clonorchiasis patients, but not with serum from healthy individual (Fig. 1A).

In an effort to identify *C. sinensis*-derived anti-allergic proteins, we evaluated the effectiveness of different *C. sinensis*-derived proteins on the secretion of granular enzyme β-hexosaminidase, a hallmark of an allergic reaction to allergen exposure, from rat basophilic leukemia mast cells (RBL-2H3). Treatment of IgE-sensitized RBL-2H3 cells with antigen (DNP-BSA) increased β-hexosaminidase secretion in a dose-dependent manner (Fig. 2B). To examine

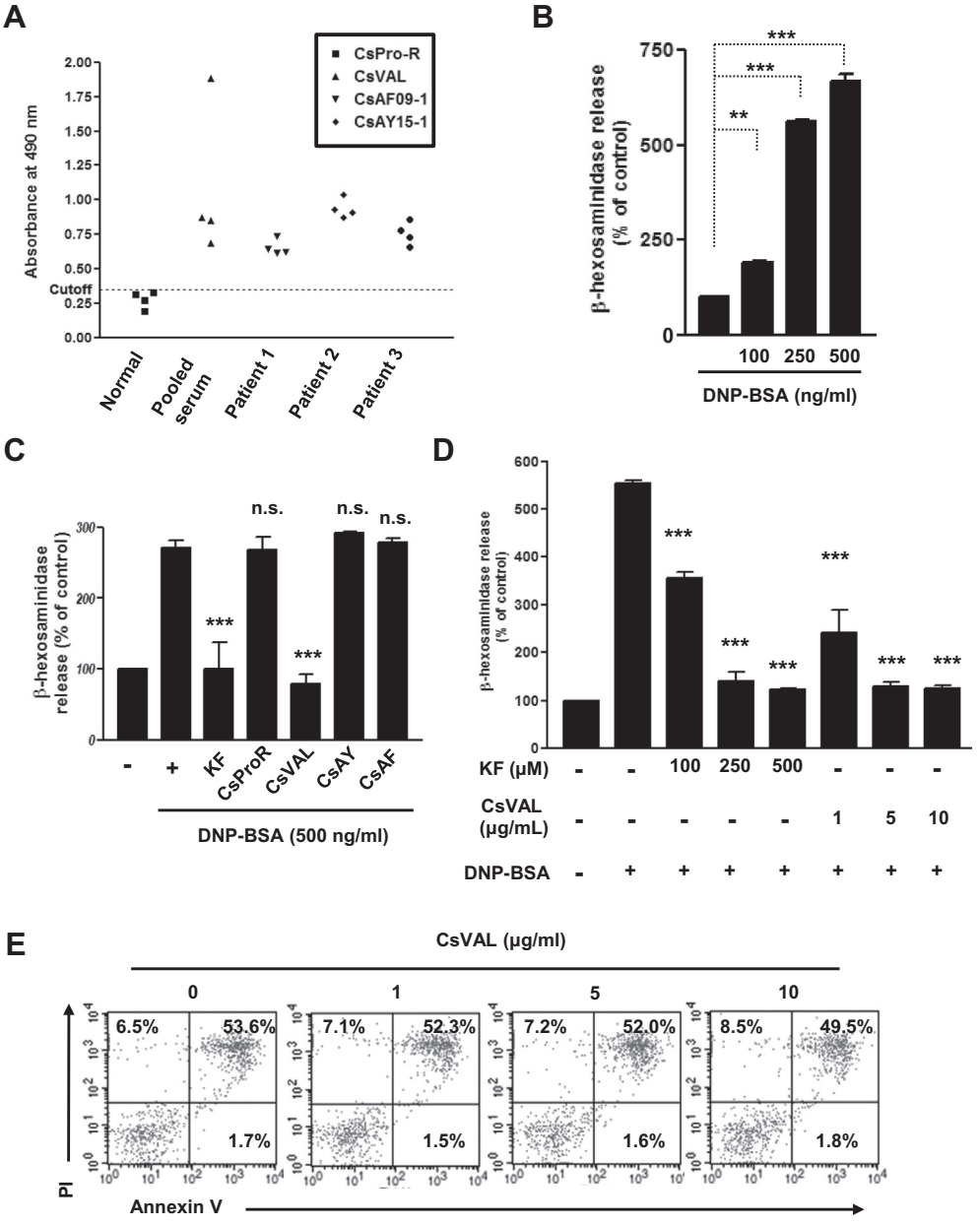

Fig. 1. Effect of CsVAL on the release of β-hexosaminidase from antigen-stimulated RBL2H3 cells. (A) Identification of antigenicity of synthetic antigenic peptides. Sera were collected from patients with clonorchiasis or healthy individuals not infected with helminths as control. Pooled sera mean the mixed serum from a number of patients (Cutoff value: 0.348807). (B) β-Hexosaminidase secretion of IgE–sensitized RBL-2H3 cells stimulated with DNP-BSA. (C) To identify *C. sinensis* antigenic peptides as possible anti-allergic candidates. Cells were preincubated for 15 min with various synthetic peptide prior to challenge with DNP-BSA. (D) The inhibitory effect of CsVAL peptide on β-hexosaminidase release. Ketotifen fumarate (KF; a positive control). Data are plotted as the mean ± standard deviation of $n = 3$ experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ was considered significantly different from DNP-BSA alone. n.s., not significant. (E) The cytotoxicity of CsVAL on mouse splenocytes. Commonly, annexin V-positive/PI-negative cells are early apoptotic (lower right quadrant), double positive cells are late apoptotic (upper right quadrant) and annexin V-negative/PI-positive cells are dead cells (upper left quadrant). These data represent five experiments.

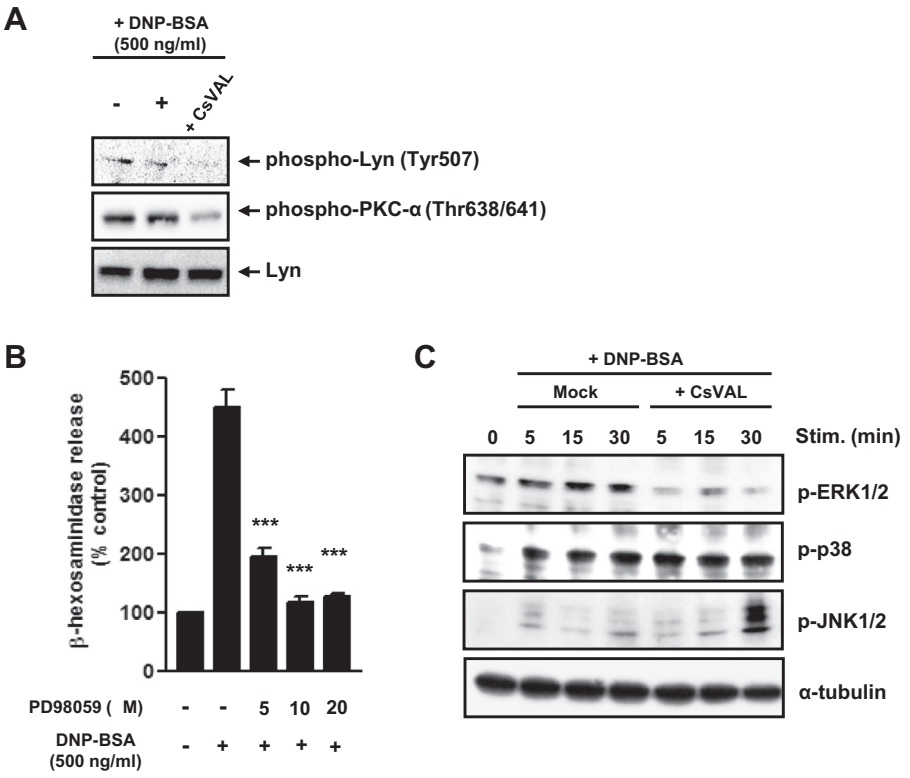

Fig. 2. Effect of CsVAL peptide on activation of FcεRI signal transduction. IgE–sensitized RBL-2H3 cells were pretreated with CsVAL (10 μg/mL) for 15 min. After stimulation with DNP-BSA, cell lysates were prepared and subjected to Western blot analysis. (A) The inhibitory effect of CsVAL on phosphorylation of Lyn (Tyr507) and PKC-α (Thr638/641). (B) Inhibition of β-hexosaminidase secretion by PD98059.$^{***}P < 0.001$ indicates statistical significance compared to DNP-BSA alone. (C) The effect of CsVAL on ERK1/2, p38, and JNK1/2 phosphorylation. α-Tubulin was detected to control for protein loading.

the inhibitory effects of various *C. sinensis*-derived synthetic peptides including CsVAL on β-hexosaminidase release, RBL-2H3 cells were sensitized with DNP-specific monoclonal IgE and preincubated for 15 min with ketotifen fumarate, an anti-allergy drug, or the individual *C. sinensis*-derived synthetic peptides prior to challenge with BSA-coupled DNP (500 ng/mL). We observed that synthetic CsVAL peptide exhibited greater inhibition of β-hexosaminidase release from antigen-stimulated RBL-2H3 cells (Fig. 1C and D). Nevertheless, the other peptides were not able to inhibit β-hexosaminidase secretion (Fig. 1C).

To confirm whether this inhibitory effect of CsVAL peptide is a non-specific response, we measured the cytotoxicity of CsVAL on mouse splenocytes by assessing changes in necrosis and apoptosis with Annexin-V and propidium iodide (PI) staining followed by flow cytometric analysis. Cells were preincubated with varying concentrations (1, 5, and 10 μg/mL) of CsVAL for 24 h prior to analysis. No marked differences were observed in the percentage of dead cells until they were incubated with 10 μg/mL CsVAL, indicating that the inhibitory effects of CsVAL on β-hexosaminidase secretion were not due to nonspecific cytotoxicity (Fig. 1E). Therefore, these results indicate that CsVAL could significantly inhibit antigen-induced mast cell degranulation.

3.2. CsVAL peptide inhibits FcεRI-mediated signal transduction in antigen-stimulated RBL-2H3 cells

The molecular consequences of mast cell activation following cross-linking of FcεRI with antigen are important for histamine release and cytokine secretion. Studies have shown that ligation of FcεRI with antigen results in activation of the Lyn, PKC, and MAPK pathways in mast cells [8,18,19]. To characterize the effect of CsVAL peptide on activation of these signaling cascades, we exposed IgE-sensitized RBL-2H3 cells to CsVAL peptide for 15 min before DNP-BSA stimulation. Examination of the Src family protein tyrosine kinase Lyn, a key signaling molecule involved in the development of allergic inflammation, revealed that pretreatment with CsVAL synthetic peptide resulted in marked inhibition of Lyn phosphorylation (Fig. 2A). Lyn activation is necessary for phosphorylation of PKC isoforms and activation of the ERK1/2, p38, and JNK1/2 MAPK pathways [8,11,20]. As expected, we observed that CsVAL significantly inhibited PKC-α activation (Fig. 2A). Moreover, we identified that inhibition of ERK1/2 by PD98059, a specific ERK inhibitor, reduced β-hexosaminidase secretion from antigen-stimulated cells (Fig. 2B). Therefore, to define whether the inhibitory effect of CsVAL synthetic peptide on antigen-induced mast cell degranulation is resulting from suppression of the MAPK family, we examined the activation of these downstream molecules in antigen-stimulated cells. Our data demonstrate that CsVAL peptide treatment inhibited ERK1/2 phosphorylation, but not influence Ag-induced levels of pp-38 and pJNK1/2 MAPK (Fig. 2C). Taken together, these results suggest that inhibition of Lyn phosphorylation by CsVAL may lead to suppression of the ERK MAPK and PKC-α phosphorylation.

3.3. CsVAL peptide prevents ROS generation in antigen-stimulated RBL-2H3 cells

Intracellular ROS induced by FcεRI cross-linking results in mast cell activation and the release of mediators such as histamine and cytokines [8,9]. Our data demonstrate an increase in ROS production in DNP-BSA-stimulated RBL-2H3 cells (Fig. 3A). This induction is inhibited by pretreatment with NAC, a general ROS inhibitor (Fig. 3A). Moreover, β-hexosaminidase release from antigen-stimulated cells was significantly inhibited by NAC treatment (Fig. 3B). These results suggest that ROS synthesis is required for optimal release of mediators from activated mast cells.

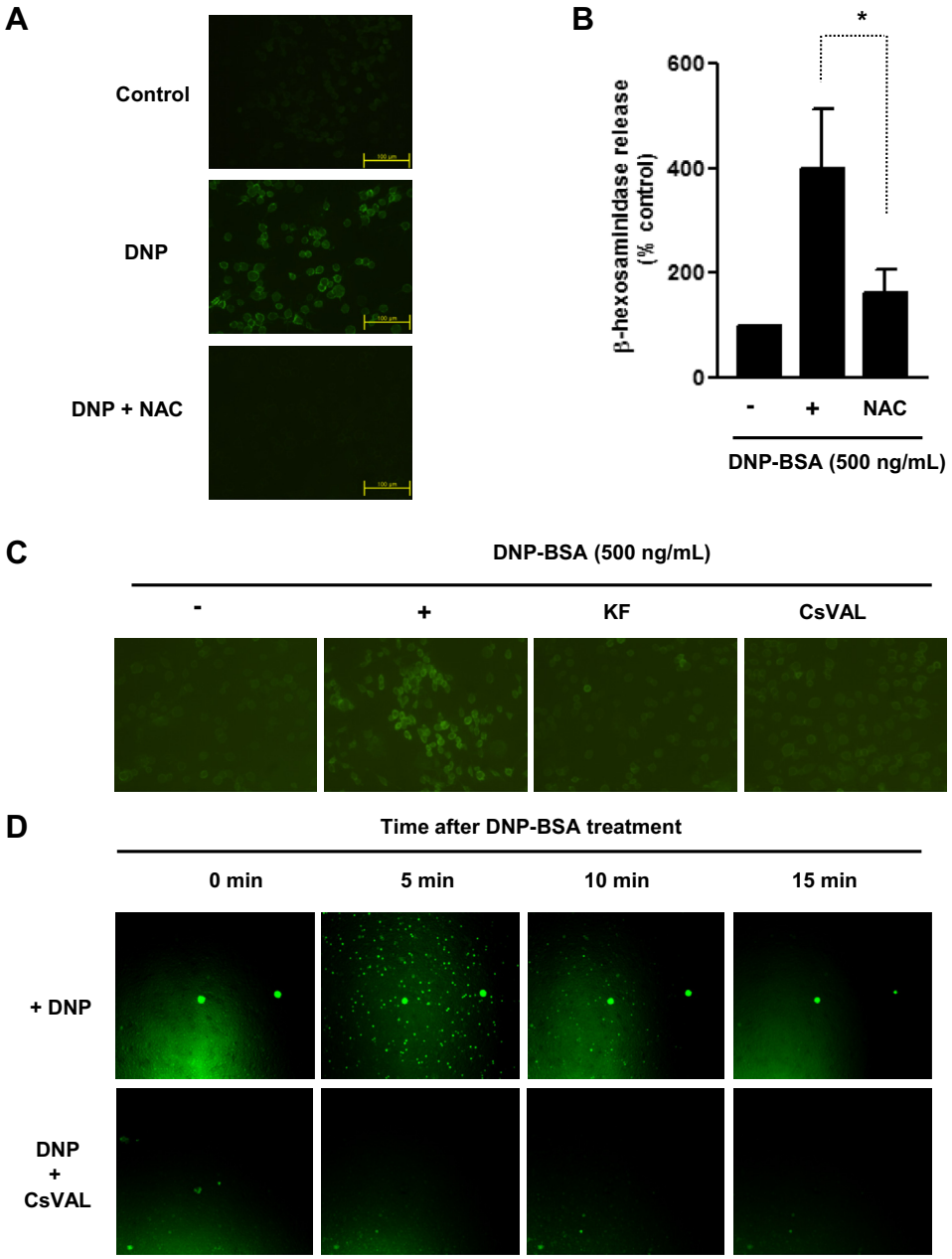

Fig. 3. Treatment of CsVAL peptides inhibits the ROS production in antigen-stimulated RBL-2H3 cells. (A) Use of fluorescence analysis to monitor intracellular ROS levels produced in antigen-stimulated RBL-2H3 cells. Pretreatment with NAC (100 μM), a general ROS inhibitor, prevents ROS formation. (B) Effect of NAC treatment on β-hexosaminidase release from DNP-BSA-stimulated RBL-2H3 cells. $^{*}P < 0.05$ indicates statistical significance compared to mock alone. (C) Sensitized cells were incubated with 10 μg/mL CsVAL peptide for 15 min before addition of DFCH-DA, after which the cells were stimulated with DNP-BSA for 5 min and observed by fluorescence microscopy. (D) Images captured by the IncuCyte™ real-time imaging system used to directly monitor changes in intracellular ROS in live cells.

To further investigate the anti-allergic effect of the CsVAL peptide, we examined whether pretreatment with the peptide could impair intracellular ROS generation in antigen-stimulated RBL-2H3 cells. Fig. 3C shows that ROS production in antigen-stimulated RBL-2H3 cells was significantly inhibited by treatment with the CsVAL peptide. Then we used the IncuCyte system to detect real-time intracellular ROS production following antigen stimulation of IgE-sensitized RBL-2H3 cells. When IgE-sensitized RBL-2H3 cells were incubated with DNP-BSA alone, the concentration of intracellular ROS increased dramatically within 5 min and then gradually decreased with time (Fig. 3D). In contrast, when IgE-sensitized RBL-2H3 cells were incubated with DNP-BSA in the presence of the CsVAL peptide, the sudden increase in ROS production was not observed. These results suggest that impairment of β-hexosaminidase release from antigen-stimulated RBL-2H3 cells induced by the CsVAL peptide occurs via a reduction in ROS production.

3.4. CsVAL peptide inhibits the chronic contact hypersensitivity response

To further investigate whether the strong inhibitory effects of the CsVAL peptide on FcεRI-mediated mast cell responses could suppress the development of allergic skin inflammation *in vivo*, we used a oxazolone-induced CHS mouse model. The CHS response to the hapten oxazolone depends on the mast cell IgE, and is accompanied by tissue edema, upregulation of inflammatory

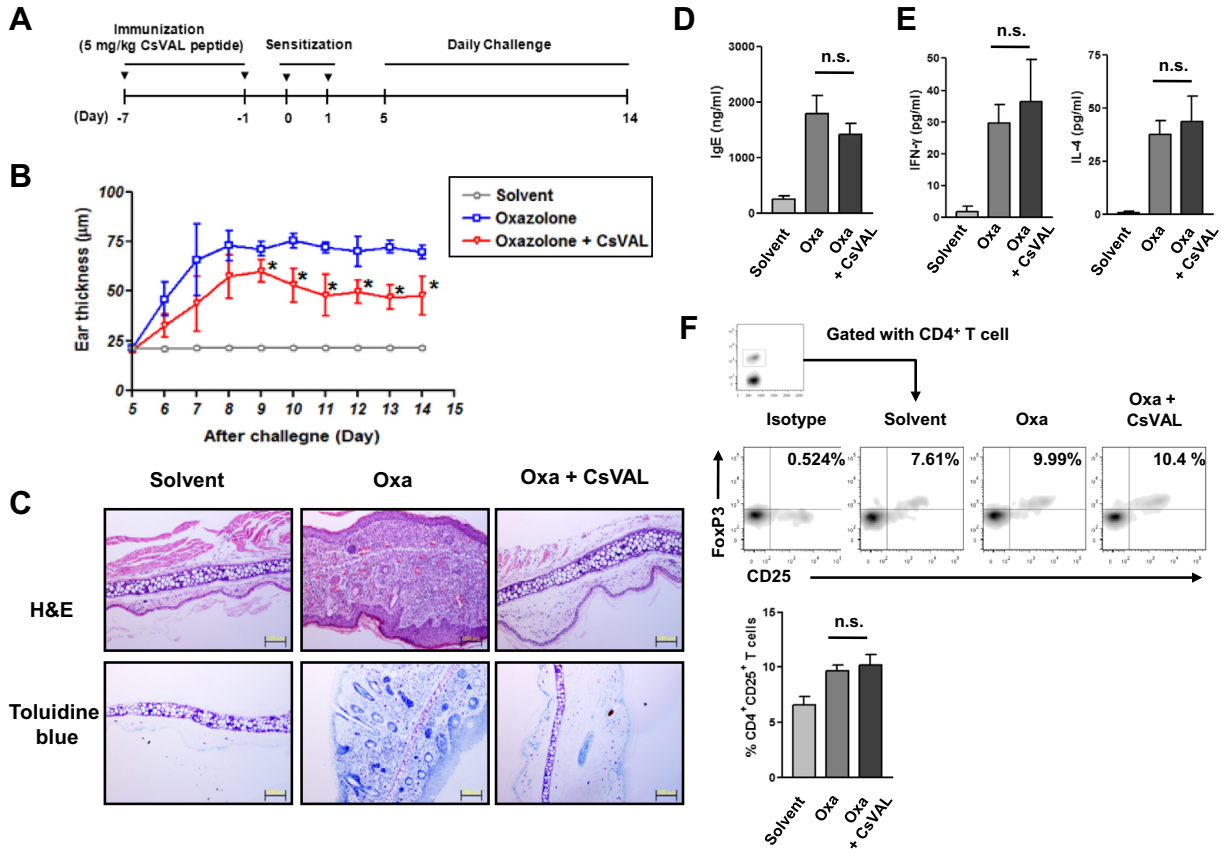

Fig. 4. Immunization with CsVAL peptide inhibits the chronic oxazolone-induced CHS. (A) Schematic depicting the schedule. The solvent control group was exposed to acetone/olive oil (4:1 v/v) throughout the duration of the experiment. (B) Ear thickness ($n = 10$ per group) was determined daily after each oxazolone challenge beginning with day 5. (C) Histological analysis reveals decreased eosinophil infiltration and acanthosis in mice immunized with the CsVAL peptide. Tissue sections were stained with hematoxylin and eosin (H&E) and toluidine blue to assess eosinophil and mast cell infiltration, respectively. (D) Total IgE levels and (E) IFN-γ and IL-4 levels in serum were measured by ELISA. (F) Flow cytometric analysis of $CD4^+CD25^+Foxp3^+$ regulatory T cells. The results are representative of three independent experiments. These *in vivo* experiments were repeated at least three times. Data represent the mean ± the standard deviation of five mice per group. Asterisks indicate statistically significant differences between mice immunized with CsVAL peptide versus oxazolone challenge alone, $^*P < 0.05$. n.s., not significant.

cytokines, and mast cell-dependent cutaneous nerve elongation [21]. To establish the chronic CHS response, mice were sensitized with 25 μl 5% oxazolone on the shaved flank skin on day 0, 10 μl 5% oxazolone on day 5, and further challenged with 2% oxazolone on the ears on days 6–14 (Fig. 4A). Oxazolone triggered a significant increase in ear thickness after initial challenge on day 5 which continued through day 14 in the CHS model, but prophylactic treatment with the CsVAL peptide inhibited this increase in ear thickness by 54.8% on day 14 (Fig. 4B). Characterization of the morphological changes in inflamed skin and cutaneous cell infiltration in the ear demonstrated abundant infiltration of granulocytes such as eosinophils and mast cells, as well as acanthosis in the dermis (Fig. 4C). There was notable improvement in eosinophil and mast cell infiltration and dermal acanthosis in mice that received a prophylactic treatment with CsVAL peptide (Fig. 4C). Serum IgE, which is enhanced in most allergic atopic dermatitis patients, was determined by ELISA as a parameter for the humoral response in Th2 conditions. However, we observed only minor suppressive effect of immunization with CsVAL peptide on IgE production at the termination time points of the CHS model (Fig. 4D). When determining the production of Th1 cytokine IFN-γ and Th2 cytokine IL-4, we could not also observed strong inhibition in the mice group immunized with CsVAL peptide (Fig. 4E). In addition, immunization with the CsVAL peptide did not affect the induction of $CD4^+CD25^+$ regulatory T cells, since the percentage of these cells remained unchanged (Fig. 4F).

4. Discussion

In the current study, we explored the inhibitory activities of the CsVAL peptide on RBL-2H3 cell activation following IgE–FcεRI interaction. We found that the CsVAL peptide effectively inhibits antigen-induced β-hexosaminidase release in IgE-sensitized RBL-2H3 cells, and may be exerted by suppressing Lyn phosphorylation and intracellular ROS generation, which are required for optimal degranulation and mast cell activation. We also found that CsVAL-induced suppression of Lyn phosphorylation and ROS generation in RBL-2H3 cells inhibits activation of PKC-α and the ERK1/2 MAPK, which are involved in histamine release and inflammatory cytokine production, respectively. A recent study that investigated the inhibitory effect of helminth-derived antigens against the development of allergic disorders also demonstrated the critical role of PKC-α in allergy [22]. That is, inhibition of PKC-α by the secreted product of a parasitic filarial nematode inhibits immediate hypersensitivity by suppressing Ca^{2+} influx and intracellular ROS production. Activation of PKC increases the level of ROS in mast cells, while inhibition of either PKC-α or PKC-δ by specific inhibitors suppresses ROS production [11]. Increased intracellular ROS production is functionally linked to mast cell activation and facilitates mediator release and Ca^{2+} influx. Therefore, our results suggest that suppression of intracellular ROS by CsVAL peptide is directly derived from inhibition of PKC-α and Lyn activation, and ultimately leads to inhibition of

β-hexosaminidase release from antigen-stimulated RBL-2H3 cells. Moreover, we found that antigen-induced mast cell degranulation is required to activate the ERK signal molecule, and the activation of this signaling molecules is inhibited by pretreatment with the CsVAL peptide. These data suggest that the inhibition of Lyn phosphorylation by CsVAL may also suppress the activation of PKC-α and ERK MAPK pathways required for the degranulation of mast cells.

We also demonstrated that these inhibitory effects of the CsVAL peptide on mast cell activation influence the suppression of the development of severe allergic skin inflammatory disease. In the CHS model, we showed immunization with CsVAL peptide suppressed skin inflammation, which is characterized by increased ear thickness and cutaneous infiltration of eosinophils and mast cells.

Interestingly, we observed that the anti-allergic effect of the CsVAL peptide appears to inhibit mast cell activation selectively. Modulation of the allergic inflammatory response associated with helminth infection may occur (i) by inducing regulatory immune cells such as regulatory T cells and regulatory B cells, (ii) by suppressing allergen-specific IgE synthesis of B cells, and (iii) by directly inhibiting FcεRI-induced release of mediators from mast cells by selectively blocking intracellular signaling pathways activated by IgE–FcεRI interaction [1,22]. Several recent reports published by our laboratory and others have demonstrated that induction of regulatory immune cells by helminth infection is important for modulating the severe allergic inflammatory response [3,23,24]. However, as supported by our data from the mouse CHS model, immunization with the CsVAL peptide had no effect on the induction of $CD4^+CD25^+$ regulatory T cells. Moreover, CsVAL peptide does not also appear inhibitory activities on the production of allergen-specific IgE and proinflammatory cytokine. In fact, treatment of U266B1 human IgE-bearing B cells and murine splenocytes with CsVAL did not affect IgE production from B lymphocytes (Fig. S1). Collectively, our findings indicate that the anti-allergic effect of the CsVAL peptide appears to inhibit mast cell effector function distinct from IgE synthesis by B cells and the development of regulatory immune cells such as regulatory T cells. In conclusion, the present study suggests that the inhibitory activities of the CsVAL peptide with regard to mast cell activation offer a new potential therapeutic approach for allergic diseases such as atopic dermatitis.

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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.bbrc.2014.01.189.

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