



Cell permeable NFAT inhibitory peptide Sim-2-VIVIT inhibits T-cell activation and alleviates allergic airway inflammation and hyper-responsiveness

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ABSTRACT

Nuclear factor of activated T cells (NFAT) is an important transcription factor for the production of interleukin (IL)-2 upon T-cell receptor (TcR) signaling. Therefore, inhibition of the NFAT–calcineurin pathway is an important target for inflammatory disease inhibition and graft rejection. A novel cell permeable peptide (CPP), Sim-2, has been identified from a human transcription factor, and Sim-2-CPP conjugated to β -galactosidase or EGFP protein was efficiently delivered into cells *in vitro* and *in vivo*. A cell permeable form of the NFAT inhibitory peptide VIVIT (Sim-2-VIVIT) was synthesized and showed inhibitory effects on human CD4 or CD8 T-cell activation through NFAT transcriptional activity suppression and IL-2 inhibition. Intranasal administration of the Sim-2-VIVIT peptide in an ovalbumin (OVA)-induced murine asthma model alleviated peribronchial and perivascular infiltration of inflammatory cells in the lung and caused airway remodeling and airway hyper-responsiveness. These results suggest that cell permeable Sim-2-VIVIT peptide has clinical potential as an immunosuppressive agent for inflammatory diseases.

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1. Introduction

T-cell activation and the induction of cytokine genes are largely attributed to a family of transcription factors, including nuclear factor of activated T cells (NFAT) [1,2]. For example, immunosuppressive drugs, such as cyclosporine A and FK506, inhibit the activity of calcineurin phosphatase leading to inhibition of NFAT-mediated interleukin (IL)-2 gene expression [3]. These drugs are potently immunosuppressive; however, they have undesired side effects, including nephrotoxicity [4] and cancer development. Thus, the development of novel immunosuppressive drugs is an important research field. To this end, the NFAT inhibitor, VIVIT, was previously developed based on the conserved calcineurin docking site in NFAT family proteins. Importantly, VIVIT peptide interferes selectively with the calcineurin–NFAT interaction without affecting calcineurin phosphatase activity [5,6].

Cell permeable peptides (CPPs), also called protein transduction domains (PTDs), have been developed to enhance delivery of therapeutic agents into many cell types [7–9]. TAT-CPP from human immunodeficiency virus (HIV-1) was firstly introduced to deliver biologically active molecules such as proteins, peptides, and si-RNA *in vivo* and has been of considerable interest as a therapeutic drug delivery tool [10–12]. Similarly, the third alpha helix of Antennapedia homeodomain [13] and the VP22 protein from herpes simplex virus [14,15] promote the delivery of covalently linked peptides into cells. Even synthetic tandem sequence of arginine (9R, 11R, etc.) peptides has potent intracellular penetrating efficiency [16,17]. In order to identify new CPPs for more clinically applicable drug delivery tool, more recently, some human-derived CPPs were identified from human proteins including Lactoferrin, Vectocell®, Hph-1, etc. [18–20] however identifying new human originated CPP sequences is still elusive.

A deregulated helper T cell-mediated immune response can cause asthma symptoms, such as pulmonary inflammation, airway eosinophilia, mucus hyper-secretion, and airway hyper-reactivity (AHR), in response to a variety of specific and nonspecific stimuli [21,22]. Previous studies have shown that the activation of Th2 CD4⁺ T helper lymphocytes is important for initiating allergic asthma and is thought to play an important role in the production of cytokines, including IL-4, IL-5, and IL-13, which stimulate and recruit macrophages, mast cells, and eosinophils [23,24].

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In this study, we evaluated the transduction activity of a novel CPP from the human transcription factor Sim-2 *in vitro* and *in vivo*. We also generated Sim-2-CPP-conjugated NFAT inhibitory peptide (Sim-2-VIVIT) and analyzed its functional activity in human T cells *in vitro* and in an *in vivo* OVA-induced asthma model.

2. Materials and methods

2.1. Generation of Sim-2-CPP-conjugated recombinant proteins

Sim-2- β -galactosidase (gal) or Sim-2-enhanced green fluorescent protein (EGFP) DNA was constructed by inserting each PCR fragment into a pRSET-B vector (Invitrogen). The BL21 (DE3) Star (Novagen) *E. coli* strain was used as the protein expression system. Recombinant protein expression was induced by 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) for 5 h, and the bacteria were harvested in lysis buffer (20 mM Tris-HCl, pH 8.0 and 500 mM NaCl). After sonication, the lysates were clarified by centrifugation (6000 rpm for 30 min at 20 °C), adjusted with 10 mM imidazole (Tris-HCl, pH 8.0), and loaded on HisTrap chelating columns (Qiagen). Bound proteins were eluted with 50, 100, and 250 mM imidazole in Tris-HCl buffer with >95% purity. The recombinant proteins contained in each fraction were desalted on PD-10 Sephadex G-25 (Amersham Pharmacia). The purified proteins were then supplemented with 10% glycerol, aliquoted, and flash-frozen at -80 °C [20].

2.2. Synthesis of the Sim-2-CPP-conjugated VIVIT peptide

Sim-2-VIVT (AKAARQAARGMAGPHPVIVITGPHEE) or control VIVIT peptides (MAGPHPVIVITGPHEE) were synthesized by AnyGen Inc. The peptides were purified by HPLC with 97% purity.

2.3. Analysis of the transduction efficiency of Sim-2-CPP *in vitro* and *in vivo*

After each cell line was incubated with Sim-2- β -gal, the cells were washed with phosphate-buffered saline (PBS) and fixed immediately. The fixed cells were then incubated with an X-gal solution for 1–3 h at 37 °C for β -gal enzyme activity. The transduction capacity of Sim-2- β -gal was also analyzed by western blot with monoclonal antibodies against β -gal (Cell Signaling Technology). Jurkat T cells were incubated with the Sim-2- β -gal proteins, and the cells were analyzed by FACS Calibur (BD Biosciences) flow cytometry. To determine the localization of the Sim-2-EGFP fusion protein, HeLa cells were incubated with 40 μ M Sim-2-EGFP fusion protein for 2 h, and the cells were fixed and stained with the DAPI for nuclear identification. EGFP fluorescence was analyzed with a fluorescence microscope (Nikon).

2.4. Analysis of T-cell activation

Jurkat cells stably transfected with the NFAT-SEAP plasmid or the AP-1-SEAP reporter gene were used to analyze NFAT or AP-1 promoter activity, and SEAP activity was measured following the manufacturer's suggested protocol (Invitrogen). Primary human CD4⁺ or CD8⁺ T cells were purified from healthy donors. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Histopaque (Sigma), the lymphocyte layer was collected, and CD4⁺ or CD8⁺ T cells were isolated using a MACS column with CD4 or CD8 microbeads (MiltenyiBiotec).

Jurkat cells (1×10^6) and primary human CD4⁺ or CD8⁺ T cells were pre-treated with Sim-2-VIVIT for 1 h, then washed with PBS, and incubated with 0.25 μ g of anti-CD28 antibodies (BD Pharmingen) for 20 min at 4 °C. The anti-CD28 antibody bound cells were further activated on a plate coated with 0.05 μ g anti-CD3 antibodies

(BD Pharmingen) for 24 h. The culture supernatants were analyzed by ELISA for the level of IL-2 production (R&D Systems).

2.5. OVA-induced experimental asthma model

Female BALB/c (Charles River Technology) mice at 6–8 weeks old were maintained under pathogen-free conditions at Yonsei Medical School. All animal experiments were performed in accordance with Institutional Animal Care and Use Committee guidelines. The experimental asthma model was modified from a previous method [25]. Mice were sensitized by intraperitoneal injection of 50 μ g OVA (Sigma-Aldrich) absorbed in 2 mg of aluminum hydroxide (Thermo) in PBS on days 0 and 14. They were subsequently challenged three times by intranasal administration of 1% OVA in PBS every day beginning on day 29. On each day of OVA challenge, mice were given 200 μ g of Sim-2-VIVIT or control VIVIT peptide 2 h before the challenge. The animals were sacrificed on day 32, and the lungs were washed *via* tracheal tube with 1 ml PBS. The differential cell counts in the bronchoalveolar lavage fluid (BALF) were determined in cytocentrifuged preparations stained with Leukostat (Fisher Diagnostics). The tissues were subjected to hematoxylin and eosin (H&E) staining for morphometric analysis and to periodic acid-Schiff (PAS) staining to measure goblet cells.

Airway hyper-responsiveness was assessed as a change in airway resistance (cm H₂O/ml/s) after challenge with aerosolized methacholine (MCh). Before sacrificing the mice, they were anesthetized with pentobarbital sodium (70–90 mg/kg, given intraperitoneal), tracheostomized (stainless steel cannula, 18 G), and mechanically ventilated. Lung function was assessed using methods described previously [25]. Lung resistance (R_L) was measured using the FlexiVent system (Scireq).

2.6. Statistical analysis

Statistical analysis of group differences was determined by the Student's *t* test. A value of **p* < 0.05 was considered significant.

3. Results

3.1. Characterization of a novel cell permeable peptide, Sim-2-CPP

A novel CPP candidate of nine amino acids (AKAARQAAR) from the human transcriptional factor Sim-2 was considered from the BLAST search screening of proteins which has comparable amino acid sequences with TAT-CPP. To determine the protein transduction efficiency of Sim-2-CPP, β -galactosidase or EGFP recombinant DNAs were constructed with or without Sim-2-CPP (Fig. 1A). The recombinant proteins were purified in bacteria as previously described [20]. To determine the transduction efficiency, the intracellular levels of proteins were analyzed after cells were treated with the proteins. In Jurkat cells, 10 μ M Sim-2- β -gal was readily transduced in a concentration- and time-dependent manner (Fig. 1B). In addition, Sim-2-EGFP or control EGFP proteins were used to analyze intracellular delivery efficiency and localization. The Sim-2-EGFP intracellular transduction efficiency was found to be concentration (Fig. 1C) and time dependent (Fig. 1D), similar to Sim-2- β -gal protein (Fig. 1B). Interestingly, levels of intracellular Sim-2-EGFP gradually reached a maximum over 7 h, suggesting that the transduction kinetics of Sim-2-CPP is different from other CPPs such as TAT or Hph-1 [26]. To examine the functional activity of intracellular β -gal protein, the enzyme activity was analyzed in HeLa and HepG2 cells. Nearly 100% of the cells became positive for β -gal enzyme activity (Fig. 1E), suggesting that Sim-2-CPP-conjugated protein has normal functional activity within the cells. To examine the intracellular localization of Sim-2-EGFP protein, the

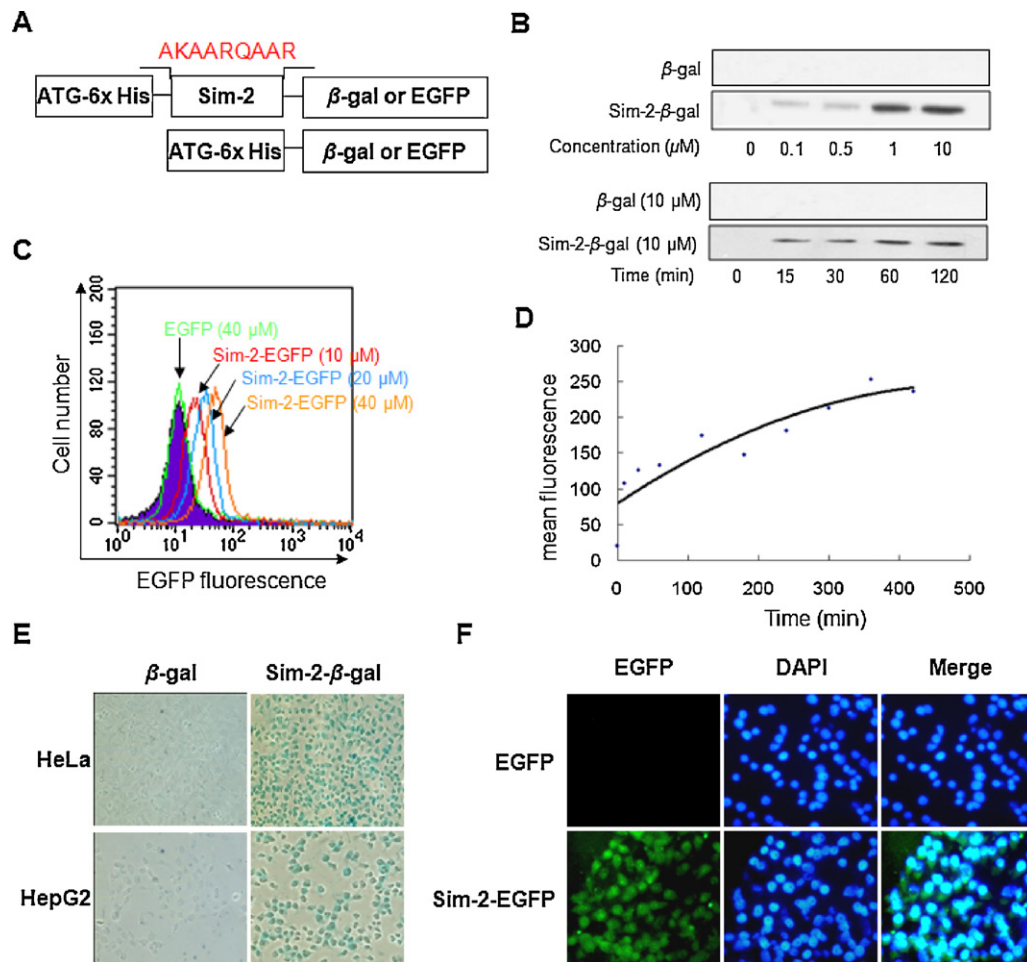


Fig. 1. Evaluation of Sim-2 as a novel cell permeable peptide. (A) DNA constructs for the Sim-2-CPP conjugated fusion proteins or control proteins. (B) β -gal proteins (0–10 μ M) were incubated with Jurkat cells for 1 h, or 10 μ M proteins were incubated with Jurkat cells for 0–120 min. Intracellular protein levels were analyzed by western blot analysis. (C) EGFP proteins with or without Sim-2-CPP were incubated with Jurkat cells at different concentrations and (D) for different time periods. Intracellular protein levels were analyzed by flow cytometry. (E) β -gal proteins (40 μ M) were pre-incubated with HeLa and HepG2 cells for 1 h. Intracellular β -gal enzyme activity was determined by X-gal solution. (F) After HeLa cells were pre-incubated with EGFP proteins for 2 h, intracellular localization of EGFP proteins was analyzed by fluorescence microscopy. The nuclei were stained with DAPI.

HeLa cells were incubated with 40 μ M protein for 2 h and the EGFP fluorescence was analyzed by fluorescence microscopy (Fig. 1F). EGFP fluorescence was visualized in the nucleus and cytoplasm, indicating that Sim-2-CPP can be used for the delivery of both cytoplasmic and nuclear molecules.

3.2. The cell permeable NFAT inhibitory peptide Sim-2-VIVIT selectively inhibits NFAT activity

By taking advantage of the capacity of Sim-2-CPP to deliver a cargo molecule into cells, the Sim-2-CPP fusion peptide, NFAT inhibitor Sim2-VIVIT, was synthesized and used to evaluate their inhibitory effects on NFAT activity upon T-cell receptor (TcR) stimulation. After pre-incubation of 500 μ M Sim-2-VIVIT or VIVIT peptide with stable Jurkat cell lines transfected with NFAT-SEAP or AP-1-SEAP DNA, the cells were activated with plate-bound anti-CD3 and soluble anti-CD28 antibodies for 24 h. Promoter activity was measured by a SEAP activity assay, and Sim-2 VIVIT was found to inhibit NFAT promoter activity significantly compared to control VIVIT peptide at 500 μ M (Fig. 2A). However, it could not affect the activity of the AP-1 promoter (Fig. 2B) suggesting that Sim-2-VIVIT specifically inhibits NFAT promoter activity.

3.3. Sim-2-VIVIT inhibits IL-2 production in activated human T cells

To examine the inhibitory effects of Sim-2-VIVIT peptide on IL-2 production from activated T cells, Jurkat T cells were pre-incubated with Sim-2-VIVIT and activated with plate-bound anti-CD3 and soluble anti-CD28 antibodies for 24 h (Fig. 3A). The production of IL-2 in the culture supernatant was inhibited by Sim-2-VIVIT, but not by control VIVIT peptide, suggesting that the inhibition of NFAT by Sim-2-VIVIT leads to the alterations in IL-2 production capacity. MACS-purified human CD4⁺ or CD8⁺ T cells from PBMCs were also used to determine the functional activity of the peptides (Fig. 3B and C). The cells without Sim-2-VIVIT or with control VIVIT peptides readily produced substantial quantities of IL-2 in response to TcR stimulation, whereas the intracellular delivery of VIVIT peptide inhibited the production of IL-2 in both primary CD4⁺ and CD8⁺ T cells. The results showed that the delivery of the VIVIT peptide into human T cells by Sim-2-CPP effectively suppressed IL-2 production by inhibiting NFAT activation. In addition, Jurkat T cell growth or survival was not affected by 500 μ M of Sim-2-VIVIT peptide (Supplementary Fig. 1A) and cell surface expression of early activation marker of CD69 was not significantly affected either by Sim-2-VIVIT and VIVIT peptide (Supplementary Fig. 1B) suggests reduction of NFAT promoter activity or inhibition of IL-2 is not due to non-specific toxic effect.

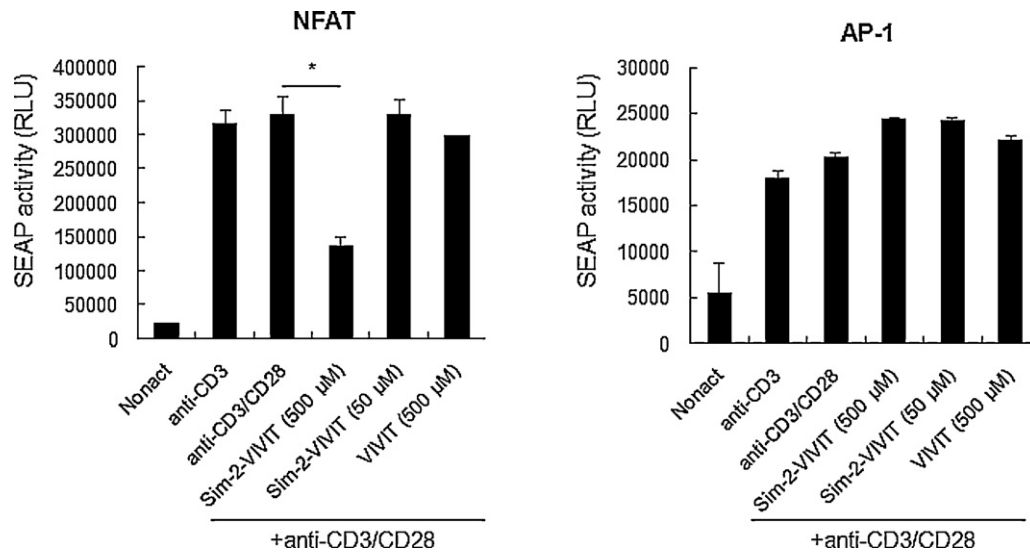


Fig. 2. A cell permeable NFAT inhibitory peptide Sim-2-VIVIT selectively inhibits NFAT activity. Sim-2-VIVIT or VIVIT peptides at 50–500 μ M were pre-incubated with Jurkat cells, which were stably transfected with (A) NFAT-SEAP or (B) AP-1-SEAP reporter plasmids for 2 h. The cells were activated with anti-CD3 and anti-CD28 antibodies for 24 h, and SEAP activity was measured. Each bar represents the mean $\pm$ SEM from three independent experiments. Student's *t* tests were used with **p* $\leq$ 0.05.

3.4. Intranasal administration of Sim-2-VIVIT peptide inhibits airway inflammation and hyper-responsiveness

To utilize the functional effect of Sim-2-VIVIT as an efficient immunosuppressant *in vivo*, we administered the peptide into an animal model of asthma intranasally. First, to determine the *in vivo* delivery efficiency, Sim-2-EGFP protein was administered intranasally, and the bronchoalveolar lavage (BAL) cells were analyzed by flow cytometry (Supplementary Fig. 2) and the frozen lung tissue was analyzed by fluorescence microscope (Fig. 4A). The fluorescent intensity in BAL cells was dose dependently increased at 20–200 μ g and 200 μ g was considered as an optimal dose for Sim-2-VIVIT in the asthma model (Supplementary Fig. 2). In the lung tissue, green fluorescence was detected in the vessel of microbronchial and endothelial area in the lung and penetrated the bronchial basement membrane. It was also localized in epithelial cells and in the tracheal basement membrane in tissue from

Sim-2-EGFP-treated mice, but was not found in control EGFP protein-treated mice (Fig. 4A). These results suggest that nasal administration of Sim-2-CPP efficiently delivers protein into the trachea and lung tissue.

To investigate the inhibitory effects of Sim-2-VIVIT peptide in allergic airway inflammation, we used the OVA-induced experimental asthma model. Before OVA challenge, 200 μ g of Sim-2-VIVIT or control VIVIT peptide was administered intranasally, and the OVA solution was nebulized to induce airway inflammation. The histological examination by H&E and PAS staining showed that OVA challenge induced the infiltration of inflammatory cells into the peribronchial tissues around the airways and pulmonary blood vessels. It also induced thickening of the airway, including increases in mucus-containing goblet cells around the bronchial airways (Fig. 4B). Remarkably, pre-treatment of Sim-2-VIVIT peptide prevented the infiltration of inflammatory cells into the peribronchial tissues, and very few goblet cells were detected

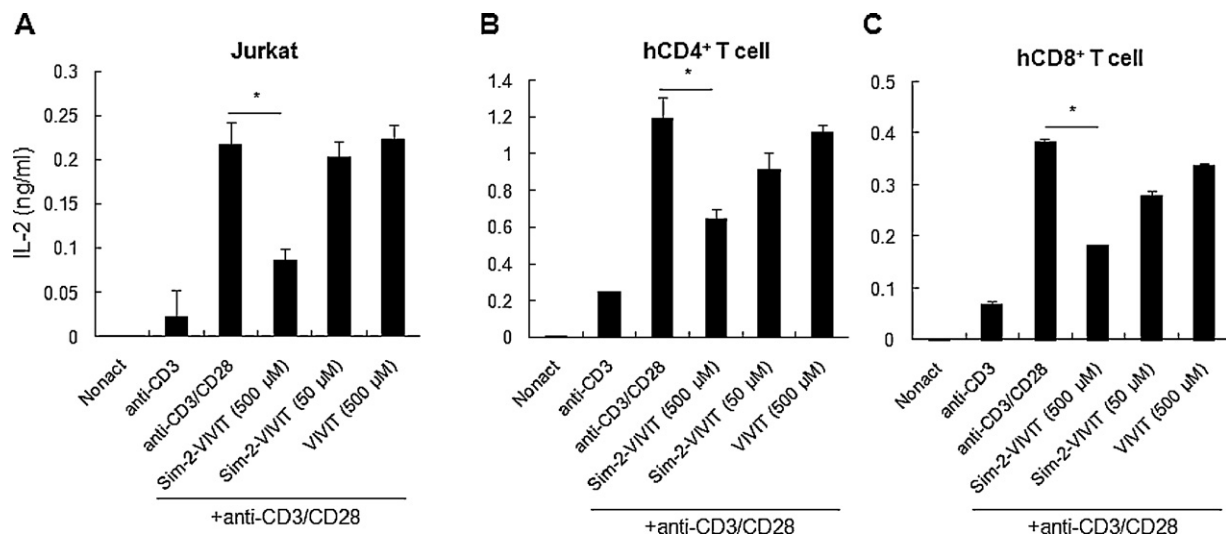


Fig. 3. A cell permeable NFAT inhibitory peptide Sim-2-VIVIT inhibits IL-2 production from activated human T cells. Sim-2-VIVIT or VIVIT peptides at 50–500 μ M were pre-incubated with: (A) Jurkat T cells, MACS-purified primary human; (B) CD4⁺ T cells, or (C) CD8⁺ T cells for 2 h, and then cells were activated with anti-CD3 and anti-CD28 antibodies for 24 h. The level of IL-2 cytokine in the culture supernatant was determined by ELISA. Each bar represents the mean $\pm$ SEM for three independent experiments. Student's *t* tests were used with **p* $\leq$ 0.05.

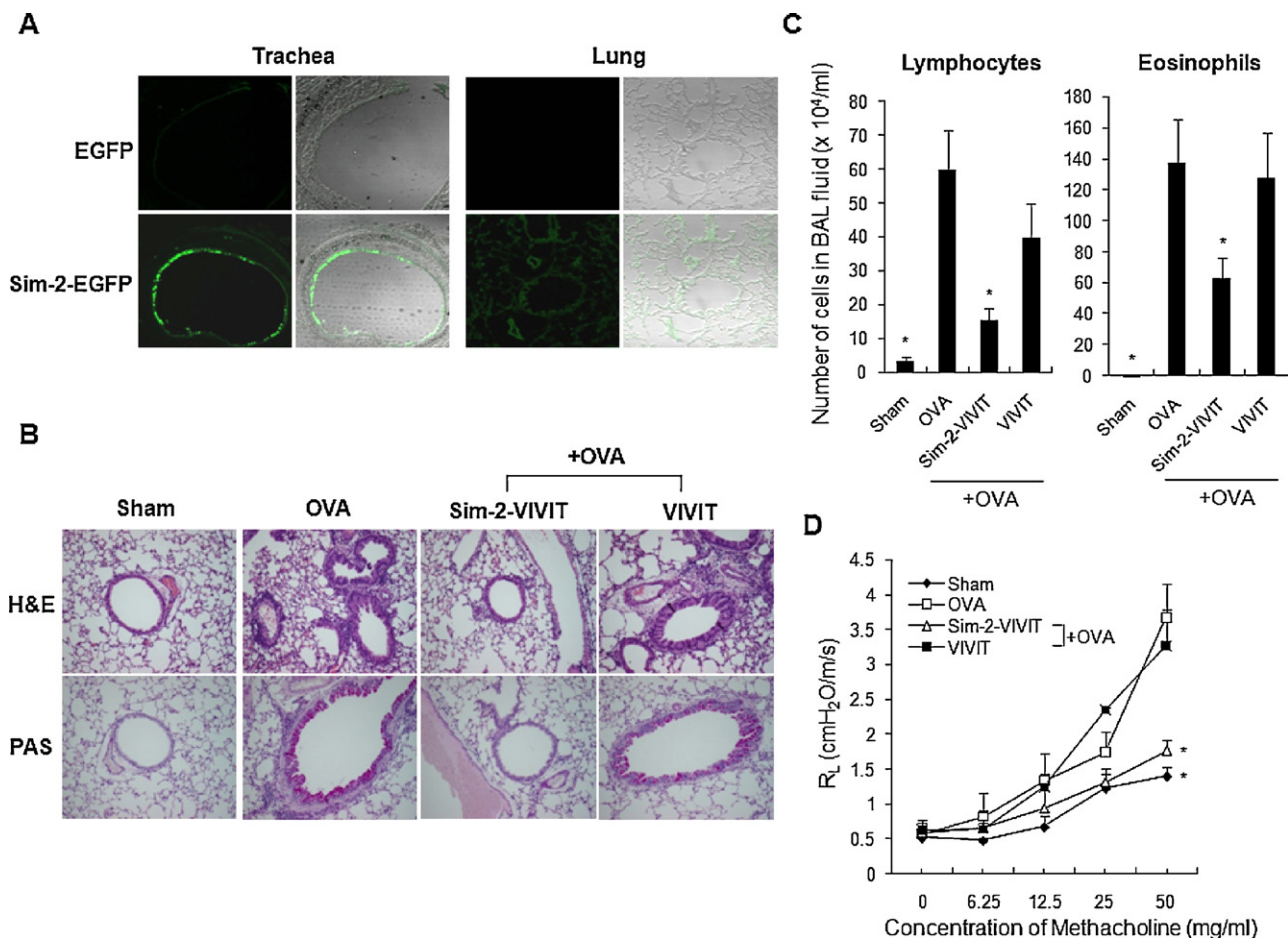


Fig. 4. Intranasal administration of Sim-2-VIVIT peptide inhibits airway inflammation and hyper-responsiveness. (A) Sim-2-EGFP or control EGFP proteins at 200 μ g were intranasally administered for 2 h. The trachea and lung tissue were then prepared for fluorescence microscopy. For airway inflammation model, the mice were sensitized and challenged as described in Section 2. (B) Lung tissue was prepared for histological analysis, including morphology, goblet cells, and the infiltration of inflammatory cells by H&E and PAS staining. (C) BAL cells were prepared, and the number of lymphocytes and eosinophils were counted. (D) Airway responsiveness was measured as the value of lung resistance (R_L) in anesthetized ventilated mice. The data represent the mean $\pm$ SEM. Student's *t* tests were used with $*p < 0.05$.

around the bronchial airways. However, significant inflammatory cell infiltration and severe mucus hyperplasia remained in the control VIVIT peptide-treated mice. The recruitment of lymphocytes and eosinophils was reduced among bronchoalveolar lavage (BAL) cells, indicating that Sim-2-VIVIT peptide significantly inhibited these inflammatory cells (Fig. 4C). These results suggest that airway inflammation was significantly inhibited by intranasal administration of the Sim-2-VIVIT peptide. In addition, airway hyper-responsiveness was evaluated by lung resistance (R_L) (Fig. 4D). The level of OVA-induced hyper-responsiveness to methacholine was substantially attenuated by Sim-2-VIVIT peptide, but not by control VIVIT peptide, suggesting that the intranasal administration of Sim-2-VIVIT peptide strongly suppressed allergic airway inflammation and airway hyper-responsiveness. These effects were likely mediated by the inhibition of T-cell activation *in vivo*.

4. Discussion

In this study, we characterized a novel cell permeable peptide, Sim-2-CPP with nine amino acids, which was designed after a human transcription factor. This peptide was administered in a murine allergic asthma model with NFAT inhibitory peptide (VIVIT). *In vitro*, Sim-2-CPP efficiently delivered conjugated proteins, and it was detected in the cytoplasm and the nucleus. Intranasal administration of Sim-2-CPP conjugated to EGFP

resulted in efficient delivery into the lung tissue, suggesting the potential use of Sim-2-CPP as a safe delivery tool for therapeutic applications.

Because the amino acid composition of Sim-2-CPP is different from that of TAT-CPP, the transduction mechanism may not same with arginine or lysine-rich sequences. The transduction kinetics of Sim-2-CPP showed a gradual increase until 7 h after administration; however, TAT or Hph-1-CPP showed rapid transduction within 15 or 30 min and reached the maximum transduction within 60–120 min [20,26]. We will further investigate the half-life of Sim-2-CPP *in vivo* compared with other CPPs. In previous reports, the cell-penetrating mechanism of TAT-CPP was suggested to be macropinocytosis [27], a type of endocytosis, and release from the endosome was indicated to be important for the functional activity of cargo molecules in the cytoplasm or nucleus. However, Sim-2-CPP seems to have different mechanism. In our preliminary experiments, intracellular localization of Sim-2-EGFP was not affected by an endosome disrupting reagent, whereas the amount of Hph-1-EGFP nuclear localization was increased by chloroquine treatment (data not shown). From these preliminary data, we suspect that Sim-2-CPP is likely transduced by a mechanism separate from macropinocytosis. Moreover, delivery efficiency of Sim-2-EGFP was not also affected by existence of 10% serum whereas that of arginine rich CPPs were inhibited by serum containing media (data now shown) suggesting Sim-2-CPP has different penetrating mechanism of cell membrane from other arginine rich CPPs.

Upon TcR stimulation, the TcR signaling pathway activates the nuclear localization of transcription factors, such as NFAT, AP-1, and NF- κ B. The immunosuppressive drugs cyclosporine A and FK506 inhibit the activity of calcineurin phosphatase, which is a substrate for NFAT. Previous reports showed that inhibition of NFAT in islet transplantation successfully inhibited graft rejection *in vivo* [16], suggesting that NFAT inhibition is an important and potent target for immunosuppression in T cell-mediated inflammatory situations. Whereas cyclosporine A and FK506 are associated with toxic side effects such as progressive loss of renal function, hypertension, hyperglycemia, neurotoxicity and increased risk of malignancy [28,29], the VIVIT peptides specifically binds with calcineurine without affecting its enzyme activity and may represent an improved calcineurine inhibitor to prevent NFAT-mediated inflammatory gene expression [16]. Previously developed drugs have harmful side effects due to their inhibitory effects on calcineurin phosphatase activity; however, VIVIT peptide interferes with the interaction between calcineurin and NFAT without affecting phosphatase activity. Thus, cell permeable VIVIT might be more advantageous and therapeutically more applicable for topical administration such as intranasal or trans-dermal delivery than current chemical immunosuppressive drugs.

In allergic airway inflammation, abnormal activation and differentiation of T cells toward the Th2 subtype is important for B-cell activation and the production of IgE and IL-4 for eosinophil recruitment [30]. Th2 cell secretes IL-13 contributes to mucus hyperplasia and the airway remodeling process [24]. Therefore, regulation of T cells to inhibit allergic inflammation has been an important research topic. Here, we demonstrated NFAT inhibition by intranasal delivery of a cell permeable form of VIVIT peptide, which dramatically prevented allergic airway inflammation and airway hyper-responsiveness in widely accepted experimental asthma model. As opposed to NF- κ B, which is associated with various signaling pathways, the NFAT transcription factor is relatively specific for the TcR signaling pathway [31]. Thus, the pathologic symptoms alleviated by Sim2-VIVIT treatment in the murine asthma model might be caused by the inhibition of T-cell activation. Multiple injection of Sim-2-VIVIT peptide for three days before OVA challenge would presumably increase the inhibitory effect on recruited T cell activation in inflammatory region. The intranasal administration of Sim-2-VIVIT is also thought to minimize potentially unexpected systemic effects.

Moreover, according to our results, Sim-2-VIVIT not only inhibited CD4⁺ T cells, but also inhibited CD8⁺ T cells, implying that this peptide may be useful for CD8⁺ T cell-mediated autoimmune or graft rejection responses. We will investigate further the function of NFAT inhibitor in CD8⁺ T cells in future studies. The current work will be expanded to novel CPPs for the delivery of other functional agents and to apply Sim-2-VIVIT to other inflammatory disease or graft rejection studies.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.imlet.2012.01.016.

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