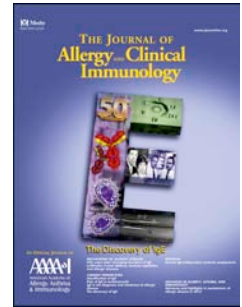


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Protein Tyrosine Phosphatase Conjugated with a Novel Transdermal Delivery Peptide, AP-rPTP Alleviates both Atopic Dermatitis-like and Psoriasis-like Dermatitis

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Protein tyrosine phosphatase conjugated with a novel transdermal delivery peptide, AP-rPTP alleviates both atopic dermatitis-like and psoriasis-like dermatitis

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Abstract

Background: Atopic dermatitis and psoriasis are the two most common chronic inflammatory skin diseases. There is an unmet medical need to overcome limitations for transcutaneous drug development posed by the skin barrier.

Objective: We aimed to identify a novel transdermal delivery peptide and to develop a transcutaneously applicable immunomodulatory protein for treating atopic dermatitis and psoriasis.

Methods: We identified and generated reporter proteins conjugated to AP, a novel transdermal delivery peptide of human origin, and analyzed the intracellular delivery efficiency of these proteins in mouse and human skin cells and tissues using multi-photon confocal microscopy. We also generated a recombinant therapeutic protein, AP-rPTP, consisting of the phosphatase domain of T cell protein tyrosine phosphatase (TC-PTP) conjugated to AP. The immunomodulatory function of AP-rPTP was confirmed in splenocytes upon cytokine stimulation and TcR stimulation. Finally, we confirmed the *in vivo* efficacy of AP-rPTP transdermal delivery in OXA-induced contact hypersensitivity, OVA-induced atopic dermatitis-like, and imiquimod-induced psoriasis-like skin inflammation models.

Results: AP-conjugated reporter proteins exhibited significant intracellular transduction efficacy in keratinocytes, fibroblasts, and immune cells. In addition, transcutaneous administration of AP-dTomato resulted in showed significant localization into the dermis and epidermis in both mouse and human skin. AP-rPTP inhibited pSTAT1, pSTAT3, and pSTAT6 in splenocytes and also regulated T cell activation and proliferation. Transcutaneous administration of AP-rPTP via the paper-patch technique significantly ameliorated skin tissue thickening, inflammation, and cytokine expression in both atopic dermatitis-like and psoriasis-like dermatitis models.

Conclusion: We identified a 9-amino acid-long novel transdermal delivery peptide, AP, and demonstrated its feasibility for transcutaneous biologic drug development. Moreover, AP-rPTP is a novel immunomodulatory drug candidate for human dermatitis.

Key messages

- A novel transdermal delivery peptide consisting of only 9 amino acids, AP, exhibited potent efficacy for the intracellular delivery of macromolecules into mouse and human skin cells and tissues.
- A novel immunomodulatory recombinant protein, AP-rPTP, negatively regulates the JAK/STAT cytokine signaling pathway, T cell activation, and T cell proliferation upon TcR stimulation.
- Transcutaneous administration of AP-rPTP alleviated tissue inflammation in both atopic dermatitis-like and psoriasis-like dermatitis mouse models, suggesting that AP-rPTP is a novel therapeutic biological drug candidate for human dermatitis.

Capsule Summary

A novel therapeutic version of protein tyrosine phosphatase with a biochemical enhancer penetrated the skin tissue barrier and ameliorated the inflammatory response in AD-like and psoriasis-like skin diseases.

Key words

Atopic dermatitis, psoriasis, transdermal delivery peptide, protein tyrosine phosphatase, TC-PTP, transcutaneous drug, immunomodulatory protein

Abbreviations

AD (Atopic Dermatitis)

- 90 AP (Astrotactin 1-derived Peptide)
91 APC (Antigen Presenting Cell)
92 CPE (Chemical Penetration Enhancer)
93 CPP (Cell Penetrating Peptide)
94 EGFP (Enhanced Green Fluorescent Protein)
95 FDC (Franz Diffusion System)
96 M β CD (Methyl-beta-Cyclodextrin)
97 OVA (Ovalbumin)
98 OXA (Oxazolone)
99 PTP (Protein Tyrosine Phosphatase)
100 SPP (Skin Penetrating Peptide)
101 STAT (Signal Transducer and Activator of Transcription)
102 TcR (T cell Receptor)
103 TDDS (Transdermal Drug Delivery System)
104 TDP (Transcutaneous Delivery Peptide)
105 TEWL (Transepidermal Water Loss)
106
107 **Conflicts of Interest** : A patent application is being prepared for the use of the transdermal
108 delivery peptide and TC-PTP in skin inflammatory diseases.
109
110

Introduction

Atopic dermatitis (AD) and psoriasis are among the most common chronic and relapsing cutaneous inflammatory diseases¹. Atopic dermatitis affects up to 25% of all children worldwide and 10% in all adults in the US^{2,3}. Psoriasis affects about 3.2% of the population worldwide, including 7.2 million adults in the US and is estimated to affect 2-4% of the population of Western countries⁴. These inflammatory skin diseases are also often related to mental health problems such as depression and suicidal ideation⁵. Moreover, the skin barrier makes drug delivery highly inefficient, resulting in a major unmet medical need for effective AD and psoriasis treatments⁶⁻¹⁰.

Both diseases involve fundamental barrier dysfunction, keratinocyte hyperproliferation, increased transepidermal water loss (TEWL), and immune cell activation and infiltration¹¹⁻¹³. T helper type 2 (Th2) cells are predominant in the acute skin lesions of AD. As the disease progresses, IL-12 secreted from infiltrated inflammatory dendritic epidermal cells (IDECs), macrophages, and eosinophils causes T helper type 1 (Th1) cells to become the predominant cell type^{1,14}. In psoriasis, Th1, Th17, and $\gamma\delta$ T cells predominate^{12,15}. Recently, Th17 and Th22 lymphocyte subtypes were suggested to be involved in both AD and psoriasis^{16,17}. Since the pathology of AD and psoriasis is mainly propagated by various inflammatory cytokine responses, modulation of cytokine signaling and/or effector T cell functions is believed to be the most promising strategy for AD and psoriasis drug development^{13,18}.

Transdermal drug delivery has many advantages in treating inflammatory skin diseases, including convenience for patients and easy dosage control^{9,10,19}. Despite extensive studies regarding transdermal drug delivery, only a few drug formulations are commercially available^{10,20}. Many transdermal drug delivery systems (TDDS) have been studied, including

chemical penetration enhancers (CPEs)⁸, microneedles^{21, 22}, iontophoresis²³, electroporation²⁴, liposomes²⁵, and nanoparticles²⁶. The general utility of previous approaches is limited by their large molecular size or other inconvenient aspects, and many cause irritation or skin damage^{19, 27}. Non-invasive and self-administered methods such as skin-penetrating peptides (SPPs) have emerged as potential solutions^{10, 27} and classical cell-penetrating peptides (CPPs) and SPPs have also been studied for possible use in transdermal drug applications. TAT²⁸, oligoarginines including R9^{29, 30}, PEP-1³¹, TD-1³², SPACE³³, and magainin³⁴ are promising vehicles for delivering macromolecules such as siRNA, peptides, and proteins. However, no FDA-approved drugs using CPPs or SPPs are currently available for macromolecular delivery, suggesting reduced efficiency in humans or unexpected side effects or toxicities in clinical trials³⁵.

In this study, we identified a novel superior transdermal delivery peptide (TDP), AP, derived from human neuronal adhesion protein (Astrotactin 1). In addition, we generated a novel immunomodulatory recombinant protein by conjugating AP to the phosphatase domain of TC-PTP (AP-rPTP). We showed that AP successfully delivered a protein into both mouse and human dermis and epidermis. Moreover, transcutaneous administration of the AP-rPTP ameliorated local skin tissue inflammation in both atopic dermatitis-like and psoriasis-like dermatitis mouse models.

Methods

For the complete description of the methods used in the *in vitro* and *in vivo* experiments, please see the Methods section in the Online Repository for this article at www.jacionline.org.

Purification of recombinant proteins. The purification protocol was modified from a previous method³⁶. For further details, see the Methods section in the Online Repository for this article.

Mice. C57BL/6 mice and BALB/c mice were purchased from Orient Bio (Daejeon, Korea). The mice were housed and bred in specific pathogen-free animal facilities at Hanyang University, Korea Advanced Institute of Science and Technology (KAIST), and Yonsei University. All mouse experimental procedures used in this study were approved by the Animal Care and Use Committees of Hanyang University, KAIST, and Yonsei University.

Human skin tissue. Normal human skin samples, with all identifiers removed, were obtained from the tissue procurement service of the Yale Department of Pathology; the Yale Human Investigations Committee declared that the use of this material was not considered human subjects research.

Statistics. Data were analyzed by two-tailed Student's t-tests, one-way ANOVA, or two-way ANOVA using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA). P values < 0.05 were considered significant.

Results

Identification of a novel skin cell-penetrating peptide, AP

With the aim of identifying novel skin cell-penetrating peptides, we performed a similarity search for peptides with multiple arginine or lysine amino acids, including TAT and R9, using the ScanProsite tool (<http://prosite.expasy.org/scanprosite/>) in the UniProtKB human database. We then filtered the results for candidates shorter than 10 amino acids. A strong candidate sequence was located in astrotactin 1, a neuronal adhesion protein. The candidate 9-amino acid of AP (177-185) is highly conserved among various species (Fig 1, A). We constructed plasmids expressing enhanced green fluorescent proteins (EGFPs) combined with or without AP and other control peptides were constructed and each protein was purified as previously described³⁶. Protein purity was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis SDS-PAGE (Fig 1, B). The purified EGFP recombinant proteins maintained their natural optical properties after peptide conjugation (Fig E1).

To analyze the skin cell-penetrating capability of the purified proteins *in vitro*, the proteins were added to the culture medium of human keratinocytes cell line (HaCaT cells) at various concentrations and for different lengths of time. Flow cytometric analysis demonstrated that the addition of increasing concentrations of AP-EGFP to the medium resulted in a dramatic increase in intracellular fluorescence (Fig 1, C). The maximal level of intracellular protein was observed at 1 hour and gradually decreased until 8 hours (Fig 1, D). In keratinocytes and fibroblasts (NIH3T3), AP exhibited significantly more efficient intracellular protein delivery than TAT-EGFP and R9-EGFP, which are the most commonly used cell-penetrating peptides (CPPs) (Fig 1, E and Fig E2). AP-EGFP exhibited robust uptake into the cytoplasm and nucleus of both keratinocytes and fibroblasts, as observed by

both fluorescence and confocal microscopic analysis (Fig 1, F and G). The fluorescence signals of TAT-EGFP and R9-EGFP were also detected with longer exposure times (Fig E3); however, their intensities were much lower than that of AP-EGFP.

Although skin cell delivery peptides have been identified using CPPs or via Franz diffusion cells (FDCs), their intracellular protein delivery efficiency is generally low in primary cells or tissues³⁵, severely limiting the clinical applications of TDDS. To further evaluate the efficiency of intracellular AP delivery into skin cells, primary epidermal cells from C57BL/6 mice were used. Mouse primary epidermal cells were isolated using dispase and trypsin as previously described³⁷ (Fig 1, H), and the intracellular fluorescence intensity of AP-EGFP was analyzed by flow cytometry. Epidermal cell types (CD45⁻ keratinocytes, CD45⁺ immune cells, CD45⁺CD3⁺ T cells, and CD45⁺CD11c⁺ antigen presenting cells) were analyzed separately, as shown in the gating strategy (Fig 1, I). 5 μ M of each EGFP protein was incubated with the isolated cells for 1 h, after which intracellular fluorescence was analyzed by flow cytometry. Significant intracellular fluorescence intensity of AP-EGFP was observed in all cellular populations, including CD45⁻ keratinocytes, T cells, and antigen presenting cells such as Langerhans cells. In contrast, little internalization of control EGFP, TAT-EGFP, and R9-EGFP was observed (Fig 1, J and E4). These results collectively suggest that AP is a novel and effective skin cell-penetrating peptide with significantly more efficient intracellular protein delivery efficiency than conventional CPPs such as TAT and R9.

Efficient transdermal protein delivery by AP into mouse and human skin tissue

Based on the potent protein delivery efficiency mediated by AP in keratinocytes and fibroblasts *in vitro*, we next determined its transdermal protein delivery efficiency *in vivo*. To enhance *in vivo* visualization, plasmids were constructed, and dTomato recombinant proteins fused to AP and other controls were generated (Fig E5). Before transcutaneous protein application, the stratum corneum was removed from each mouse using depilatory cream and tape stripping (see Methods section for further details). On the dorsal skin of GFP transgenic mice, 100 μ g of dTomato and AP-dTomato was applied for 6 h via the paper patch method. As shown in Fig 2A, significant AP-dTomato fluorescence signal was observed in both the dermis and epidermis with overlapping GFP-positive skin cells in the tissue, while the dTomato protein signal remained on the skin surface (Fig 2, A and B). In addition, the dermal fluorescence signal intensities of TAT-dTomato and R9-dTomato were much weaker than that of AP-dTomato. (Fig 2, C).

For better visualization of AP-dTomato transdermal delivery, we performed multi-photon microscopic analysis in live mice. Proteins were administered by the paper patch technique for 6 h, after which anesthetized mice were prepared under the microscope. Significant AP-dTomato fluorescence was observed at a skin depth of 40-120 μ m, while R9-dTomato was predominantly present at a depth of 60 μ m, and TAT-dTomato was mostly retained on the skin surface (Fig 2, D and E). Z-projection data from 40 μ m to 120 μ m revealed accumulated fluorescent intensity in the sample tissue, suggesting that the total amount of dermis skin penetrated by dTomato was highest when conjugated to AP and was also greater than that of any of the other controls (Fig 2, F and G). For quantitative analysis, significant fluorescence signals over the threshold were represented as spheres. The spheres were counted by Imaris software. This analysis revealed that significantly more protein was

detected in the dermis of AP-dTomato treated mice than in that of other controls. (Fig 2, H and I).

Next, we determined the transdermal delivery efficiency of AP-dTomato protein into isolated human skin tissue. We applied 200 μ g of protein via the paper patch method onto isolated human breast skin tissue samples for 12 h. For this incubation, the samples were placed on gauze and medium (Fig 3, A and B). The human breast skin tissue samples were procured as full thickness specimens from healthy donors and exhibited much thicker epidermis and dermis than those of mouse skin tissue (Fig 3, C). Using these human breast skin tissue samples from three different donors, we performed *in vitro* transcutaneous protein delivery experiments. Significant intracellular AP-dTomato signal was observed in both the dermis and epidermis of the human skin tissue samples, indicated with a white arrow, based on colocalization with a nuclear stain. However, the signal of dTomato protein without any peptide conjugation was confined to the skin surface, and little intracellular signal of TAT-dTomato was observed (Fig 3, D). These results demonstrate that AP is an efficient skin tissue-penetrating peptide that enables transcutaneous protein delivery into the dermal and epidermal cells of both mouse and human skin.

Skin cell penetration mechanism of AP

The superior skin tissue penetration and intracellular delivery efficiency of AP-conjugated proteins prompted us to investigate the mechanisms by which the AP peptide mediates protein delivery. We hypothesized that the intracellular protein delivery mechanism to skin cells would mainly involve endocytosis, like other conventional CPPs. To test this hypothesis, the intracellular fluorescence intensity of AP-EGFP in HaCaT cells was determined after incubation with various inhibitors such as heparin³⁸, methyl-beta-cyclodextrin (M β CD)³⁹, amiloride⁴⁰, or chlorpromazine⁴¹, each of which could potentially interfere with the intracellular transduction of CPPs. The skin cell-penetrating efficiency of AP-EGFP was strongly inhibited by heparin (Fig 4, A), M β CD (Fig 4, B) and amiloride (Fig 4, C), but not by chlorpromazine (Fig E6A). These results suggest that recombinant AP-fusion proteins are internalized by lipid raft-mediated endocytosis and/or macropinocytosis following heparan sulfate interaction. Conversely, the decreased internalization efficiency observed at low temperatures or in the presence of serum-containing medium (Fig E6B and C) suggests that AP-EGFP is internalized by ATP-dependent and protein interaction-dependent mechanisms. These results suggest that the internalization mechanism of AP-EGFP consists predominantly of endocytosis initiated by heparan sulfate interaction, like other CPPs.

Next, we investigated mutant versions of AP to determine the amino acids critical for its potent cell penetrating capability. Since positively charged amino acids are key amino acids for interactions with proteoglycan⁴², we first generated arginine-deleted versions of AP-EGFP, including AP(Δ R₁)-EGFP (RRWCKRRR), AP(Δ R₉)-EGFP (RRRWCKRR), and AP(Δ R₁ Δ R₉)-EGFP (RRWCKRR) and determined their intracellular delivery efficiency in HaCaT cells (Fig 4, D). A single arginine deletion at either the N-terminal or C-terminal dramatically reduced intracellular protein delivery, suggesting that three arginine amino acids

at both ends is the optimal number for protein transduction. We next generated single mutants of the non-arginine amino acids in the AP sequence (W, C, and K). The cysteine mutant AP (APCA) showed the most dramatic decrease in transduction efficiency, while the tryptophan mutant (APWA) and lysine mutant (APKA) exhibited significantly reduced intracellular protein delivery (Fig 4, E). We also hypothesized that adding a cysteine residue to other CPPs, such as TAT, LPIN, and NP2, would increase their intracellular delivery efficiency. All CPPs with an additional cysteine residue appended to the C-terminal end of the sequence showed significantly increased in transduction efficiency in HaCaT cells (Fig 4, F). These results collectively suggest that each amino acid in AP contributes to its potent skin cell penetrating capability, and that the composition of arginine, lysine, cysteine, and tryptophan is the optimal combination.

Recombinant TC-PTP conjugated to AP is a novel skin cell-penetrating immunomodulatory molecule

Regulation of cytokine signaling is considered to be an ideal strategy for developing drugs intended to modulate the local inflammatory response in skin tissue¹⁸. T cell protein tyrosine phosphatase (TC-PTP/PTPN2) is an enzyme of the protein tyrosine phosphatase (PTP) family that is an attractive drug target for regulating the JAK-STAT cytokine receptor signaling pathway. This pathway includes JAK1⁴³, JAK3⁴³, STAT1⁴⁴, STAT3⁴⁴, STAT5⁴⁵, and STAT6⁴⁶. TC-PTP also inhibits T cell receptor signaling molecules such as Src family tyrosine kinases (Lck, Fyn)⁴⁷. We hypothesized that AP-conjugated TC-PTP would be an effective immunomodulator in skin inflammatory diseases. To test this hypothesis, we generated DNA constructs (Fig 5, A) and used them to express and purify truncated versions of TC-PTP containing the catalytic domain and substrate binding site fused to the AP sequence (AP-rPTP) (Fig 5, B). The 3D-structure of AP-rPTP was predicted by the structure modeling tools Sparks^X and visualized by PyMOL. This analysis revealed that the recombinant fusion protein was expected to exhibit no significant changes compared to its original structure (Fig 5, C). We further confirmed the phosphatase activity of purified recombinant AP-rPTP using a chromogenic substrate. This analysis revealed that the AP-rPTP exhibited significant enzymatic activity (Fig 5, D).

We next confirmed the intracellular delivery efficiency of AP-rPTP into HaCaT cells. For these experiments, AP-rPTP was used at a concentration of 1 or 5 μ M and cells were incubated with fusion protein for 1 hour. Flow cytometry analysis with an anti-6xHis-tag antibody revealed that the fusion protein was significantly delivered into cells in a concentration-dependent manner (Fig 5, E). The intracellular levels of AP-rPTP reached a maximum at 2 h and then gradually decreased until 12 h (Fig 5, F). We next determined whether AP-rPTP inhibits cytokine signaling in stimulated cells. To this end, mouse

splenocytes were treated with AP-rPTP or AP-EGFP for 1 h. The cells were then stimulated with IFN- γ , IL-6, and IL-4 for 60 min, 15 min, and 30 min respectively (Fig 5, G). AP-rPTP significantly reduced STAT1, STAT3, and STAT6 phosphorylation, suggesting that AP-rPTP negatively regulates cytokine signaling in cells. We next determined the regulatory effects of AP-rPTP on T cells by stimulating FACS-sorted naïve CD4 T cells with anti-CD3 and anti-CD28 antibodies for three days, after which proliferating cells were analyzed by the CFSE assay. AP-rPTP treatment significantly reduced activated T cell proliferation (Fig 5, H). Moreover, the production of various cytokines (IL-2, IFN- γ , IL-13, and IL-17) by mouse splenocytes activated with anti-CD3 and CD28 antibodies was also significantly reduced by AP-rPTP (Fig 5, I). These results suggest that recombinant AP-rPTP is a potent regulatory molecule that inhibits both inflammatory cytokine signaling, T cell activation, and T cell proliferation.

Transdermal administration of AP-rPTP inhibits atopic dermatitis-like skin inflammation

To determine whether transdermal administration of AP-rPTP protein reduces skin inflammation, we used oxazolone (OXA)-induced contact hypersensitivity (CHS) and OVA-induced atopic dermatitis-like mouse models. For the CHS model, mice were prepared for hair removal on day 0. On day 1, after initial sensitization with 2% OXA, 100 µg of proteins was applied on each side of the ear via the paper patch method every day for 6 days. The attached paper patches were firmly maintained by water surface tension for 1 h on ear. Ear thickness was measured every day; on day 6 mice were challenged with 1% OXA and skin tissue histology and cytokine mRNA analyses were performed 24 hours thereafter (Fig 6, A). Ear thickness was significantly increased in the AP-EGFP-treated group; however, transcutaneous delivery of AP-rPTP by paper patch significantly inhibited ear thickening (Fig 6, B), as demonstrated by the reduced weight of the ear skin biopsy samples (Fig 6, C). Moreover, visual observation revealed that skin dryness was significantly improved in the AP-rPTP-treated tissue (Fig 6, D). Histological H&E staining of the skin tissue samples revealed significant cellular infiltration of neutrophils, eosinophils and lymphocytes into the ear and marked epidermal hyperplasia in the AP-EGFP-treated group, while AP-rPTP-treated tissue exhibited significantly reduced pathologic signs (Fig 6, E, Fig E7A and B). We observed similar results in a back skin CHS model (Fig E8). Importantly, neither TAT-rPTP nor R9-rPTP inhibited this inflammatory response, suggesting that AP-mediated skin tissue penetration is critical for rPTP-mediated regulatory functions (Fig E9A and B). In addition, the mRNA levels of the pro-inflammatory cytokines IL-1 β and IL-6 (Fig 6, F) and the mRNA levels of the chemokines CXCL2 and CXCL5 (Fig 6, G) were significantly reduced by AP-rPTP treatment. Cumulatively, these data suggest that AP-rPTP regulates OXA-induced contact hypersensitivity.

Next, we utilized an atopic dermatitis-like mouse model with the OVA antigen. To this end, 100 μ g of OVA was applied to sterile gauze on the dorsal skin of BALB/c mice for one week, after which the protein was removed. This procedure was repeated four times at 2-week intervals. For the prevention group, 100 μ g AP-rPTP was co-administered with OVA during all four sensitizations. For the therapeutic group, AP-rPTP was co-administered with OVA only in the last two sensitizations (Fig 6, H). Histological analysis (H&E staining) of inflammatory skin tissue from mice in the OVA+PBS group revealed significant epidermal hyperplasia, edema, and substantial cellular infiltration; however, transdermal delivery of AP-rPTP significantly reduced inflammatory symptoms and epidermal thickness in both the prevention and therapeutic groups (Fig 6, I, J and K). Moreover, significantly fewer neutrophils and eosinophils had infiltrated in the AP-rPTP treated group (Fig 6, L, M and Fig E10). Analysis of infiltrated mast cells and T cells, which were stained with toluidine blue and anti-CD3 antibodies respectively (Fig 6, N and O), revealed that mice in the AP-rPTP treated groups exhibited fewer mast cells and T cells than mice in the control group (Fig 6, P and Q). In addition, the IL-13 mRNA level in skin tissue was significantly decreased in both the prevention and therapeutic groups, suggesting that Th2 inflammation is reduced by the corresponding treatments (Fig 6, R). After the last OVA sensitization, transepidermal water loss (TEWL) of the skin was also measured. This analysis revealed that TEWL was significantly decreased in the AP-rPTP treated groups, suggesting that transdermal delivery of AP-rPTP improves skin epithelial cell function (Fig 6, S). These data collectively suggest that transcutaneous administration of AP-rPTP by the paper-patch technique significantly inhibits contact hypersensitivity and atopic dermatitis-like skin inflammatory diseases.

Transdermal administration of AP-rPTP inhibits psoriasis-like skin inflammation

To determine whether AP-rPTP could regulate other type of inflammatory skin diseases like psoriasis, we examined the effects of AP-rPTP in imiquimod-induced psoriasis-like dermatitis model. For this model, mice were prepared for hair removal on day 0. Specifically, 70 μ g of AP-rPTP and the control protein was applied on each side of the ear for 1 h by the paper-patch technique. Next, a mixture of 20 mg Aldara cream (5% imiquimod) and an additional 30 μ g of protein was applied for 6 consecutive days, after which disease pathology was analyzed on day 7 (Fig 7, A). The ear thickness of AP-EGFP-treated mice increased significantly from day 2 to 7 of imiquimod treatment, while transdermal AP-rPTP treatment significantly inhibited this ear thickening (Fig 7, B), inflammatory cellular infiltration (Fig 7, C), and epidermal hyperplasia (Fig 7, D). Moreover, fewer infiltrated lymphocytes and neutrophils were observed in the AP-rPTP group (Fig 7, E). The ear skin tissue mRNA expression levels of IL-17A, IL-17F, and IL-6 were dramatically reduced by AP-rPTP administration (Fig 7, F). Furthermore, significant reduction of S100A8/9 (Fig 7, G), a mediator of neutrophil chemotaxis and the adhesion effect, was observed. We also performed similar experiments on mouse back skin, which has thicker epidermis and dermis than ear skin. Transcutaneous treatment of back skin with AP-rPTP also significantly prevented imiquimod-induced dermatitis signs such as skin thickening, scaling, and erythema (Fig 7, H and I). Histological analysis of the AP-rPTP treated group revealed fewer infiltrated immune cells and elongated rete ridges (Fig 7, J) and reduced epidermal thickness (Fig 7, K) than in the AP-EGFP group. These results collectively suggest that transdermal administration of AP-rPTP via the paper-patch technique efficiently regulates psoriasis-like inflammatory skin disease.

Discussion

In the present study, we described a novel transdermal delivery peptide, AP, derived from human astrotactin 1. We showed that this peptide exhibits superior delivery efficiency in both mouse and human skin. In addition, conjugation of AP to the catalytic domain of T cell protein tyrosine phosphatase (AP-rPTP) resulted in a protein with immunomodulatory functions in cytokine signaling and T cell activation and proliferation. Finally, we demonstrated that AP-rPTP is a novel therapeutic protein that can be applied transcutaneously in atopic dermatitis and psoriasis.

Atopic dermatitis (AD) and psoriasis are very common inflammatory diseases^{3, 4}. The only available topical treatments for regulating inflammation are corticosteroids, which suppress various inflammatory pathways⁴⁸, and tacrolimus and pimecrolimus, which target calcineurin binding to FK506-binding protein (FKBP12)⁴⁹. These small molecule ointments are currently used as topical therapeutics, although they have various efficacy and safety limitations⁵⁰. Patients with severe symptoms have also been treated with systemic immune-suppressants such as cyclosporine A or corticosteroids⁵¹, although a number of undesirable systemic side effects have been reported including hypertension, nephrotoxicity, and gastritis⁵¹. Therefore, there is a significant unmet need for the development of a safe and effective drug for AD and psoriasis⁶. The stratum corneum, which forms the outermost layer of the epidermis, hinders penetration of the skin barrier by macromolecules⁹. Since only small molecules (under 500 Dalton) can penetrate the stratum corneum, cyclosporine A (CsA) (1,202 Da) was not effective via transcutaneous administration for atopic dermatitis^{7, 52}. With the aim of overcoming this size limitation, CsA was fused to hepta-arginine (R7-CsA); transcutaneous administration of this peptide reduced ear inflammation in a contact

hypersensitivity model³⁰. This result implies that CPPs could potentially be used for transcutaneous drug delivery.

In 1979, the scopolamine patch (used for motion sickness) became the first US FDA-approved transdermal drug. Transdermal drug delivery systems (TDDS) have since been extensively developed, with 39 drugs approved as of 2014²⁰. TDDS are classified into three generations, as defined by Prausnitz et al¹⁰. First generation topical formulations such as patches, gels, and liquid sprays comprise the majority of currently approved transdermal drugs on the market. This system is more attractive than oral delivery, but the drug candidates must be small, lipophilic, and efficacious at low doses. Second generation systems such as chemical enhancers (CPEs)⁵³, iontophoresis⁵⁴, and noncavitational ultrasound⁵⁵ increase skin permeability or provide a driving force for delivery. Although these systems successfully enhance delivery efficacy, they can also cause irritation, pain, and tissue damage. The third generation of drugs includes CPEs, biochemical enhancers, electroporation, cavitational ultrasound, microneedles, thermal ablation, and macromolecule delivery through tools such as vaccines. The microneedle system is becoming more popular, and many approaches are currently being tested in clinical trials⁵⁶. Microneedles have mainly focused on vaccine delivery, insulin delivery, or cosmetic applications due to their limitations for intracellular delivery^{21, 22}. Biochemical enhancers such as skin tissue-penetrating peptides (SPPs), which are composed of a few amino acids and can be conjugated to various cargo macromolecules, have recently been shown to be safe and effective enhancers for regulating skin cell behavior.

Various SPPs have been developed. These SPPs can be classified into two main groups: traditional cell-penetrating peptides (CPPs; e.g. TAT, oligoarginine, and PEP-1) and skin tissue penetrating peptides (SPPs; e.g. TD-1 and SPACE)²⁸⁻³³. Cyclopeptide TD-1 has been used to deliver insulin without covalent linkage to suppress serum glucose levels³². Cyclosporine A, FK506BP, and siRNA have been delivered using oligoarginine, Pep-1, or

465 TAT to treat dermatitis in a mouse model^{28, 30, 31}. However, several limitations were reported
466 for these peptides. For instance, TD-1 transports cargo via the transfollicular route, which is
467 less relevant for treating humans because hair follicles are rare on human skin. Moreover,
468 oligoarginine was not efficiently delivered to viable epidermal and dermal cells without
469 pyrenebutyrate, a hydrophobic counteranion; ; furthermore, SPACE peptide-conjugated cargo
470 did not execute their proper functions in a disease model^{29, 32, 33}. In our experiments, the
471 MTD-1067 and S3-2 peptides, which were screened by phage display, were unable to deliver
472 cargo protein into skin cells (Fig E11). In addition, we found that TAT-rPTP and R9-rPTP did
473 not prevent the development of dermatitis and only AP-rPTP could suppress ear
474 inflammation. Although several previous studies have demonstrated successful
475 transcutaneous drug delivery in animal models of skin disease, no clinically available topical
476 therapeutics that use biochemical enhancers are yet available, posing possible limitations for
477 their clinical use³⁵. This limitation might be due to discrepancies between animal model skin
478 and human skin. For instance, mouse skin which is commonly used for cutaneous permeation
479 studies and mice are common disease models, but mouse skin is significantly thinner than
480 human skin. We found that AP-rPTP could modulate back skin inflammation as well as ear
481 skin inflammation in CHS, atopic dermatitis-like, and psoriasis-like models. In addition, the
482 application of 200 µg of AP-dTomato onto isolated human skin tissue samples showed
483 successful intracellular localization in both the epidermis and dermis. This localization was
484 rarely observed in the TAT-dTomato-treated samples, suggesting that AP-dTomato has a
485 higher potential for clinical usage than TAT. We also performed hair removal and tape
486 stripping and showed that AP-dTomato was significantly delivered into the intact skin tissue,
487 as demonstrated by the more even distribution in the dermis following tape stripping (Fig
488 E12A). In dermatitis disease models, AP-dTomato treatments without tape stripping resulted
489 in more efficient delivery of AP-dTomato into the dermis of inflammatory tissue compared to

control treatments (Fig E12B). Thus, we suggest AP is a significantly improved third generation TDDS.

Intracellular protein delivery by AP involves lipid-raft mediated endocytosis and macropinocytosis following heparan sulfate interaction, a mechanism similar to that of other CPPs. Phagocytic cells uptake CPP-proteins better than lymphocytes and activated or memory like lymphocytes have better endocytosis ability than resting lymphocytes suggesting heterogeneity of CPP-mediate protein delivery efficiency^{57, 58}. Here we also consistently demonstrate CD11c positive antigen presenting cells or CD45 negative keratinocytes have higher intracellular AP-EGFP signal than CD3 positive cells. Interestingly, cysteine is a critical amino acid that contributes to the superior cellular uptake of AP-conjugated proteins. AP dimerization is mediated by its cysteine residue, thereby enhancing binding affinity to the cell membrane compared to the monomeric form of AP and providing entropy advantages⁵⁹. The transfection efficacy of plasmid DNA has been shown to be enhanced by appending a C-terminal cysteine to penetratin and PenArg (a penetratin analog) and also to the EB1 sequence; this enhancement was mediated by dimerization⁵⁹. CyLoP-1, a cysteine-rich peptide derived from the natural cationic polypeptide toxin crotoxin, exhibited reduced delivery efficiency after its cysteine residue was substituted to a serine residue or deleted,⁶⁰ suggesting that cysteine enhances cellular penetration efficiency. We previously reported various human derived cell-penetrating peptide such as LPIN⁶¹, 2IL-1 α ⁶², dNP2³⁶. By adding one cysteine to LPIN, it showed significantly increased protein delivery efficacy than LPIN. dNP2 is a tandem repeat of NP2 sequence³⁶. By adding a cysteine to NP2, it also exhibited significantly better delivery efficiency than NP2 suggesting cysteine is important for superior efficiency of AP. Tryptophan is commonly found in membrane surface proteins. The aromatic group of this amino acid creates non-covalent interactions with regions of π -electron density^{63, 64}. Within basic charged amino acids, tryptophan induces

glycosaminoglycan (GAG)-dependent endocytosis via the formation of the β -strand structure complex with GAGs⁶⁴. Therefore, the tryptophan residue in AP also contributes to interactions with the cell membrane. This combination of functional amino acids in AP enhances intracellular drug delivery.

TC-PTP is a non-receptor-type protein tyrosine phosphatase encoded by the *Ptpn2* gene that has been reported to negatively regulate cytokine and TcR (T cell receptor) signaling⁴⁷. Polymorphisms in TC-PTP have been widely reported in patients with various autoimmune diseases, but few studies have investigated the role of TC-PTP in cutaneous inflammatory diseases⁶⁵. However, spontaneous inflammation in the skin has been observed in CD4-specific TC-PTP knock out mice⁴⁴. TC-PTP also regulates various cellular signaling pathways, including the Src family (involved in TcR signaling⁴⁷) and the JAK-STAT pathway (including its members JAK1⁴³, JAK3⁴³, STAT1⁴⁷, STAT3⁴⁷, STAT5⁴⁵, and STAT6⁴⁶). Inflammatory cytokines that mainly use the JAK-STAT pathway induce inflammation and barrier impairment⁶. Various cytokines secreted from keratinocytes and T cells, including TSLP, IFN- γ , IL-4, IL-5, IL-13, IL-22, IL-31, and IL-33, have a crucial role in AD pathogenesis⁶. Furthermore, TNF- α , IFN- γ , IL-6, IL-22, and IL-17, which are secreted from keratinocytes, helper T cells, and $\gamma\delta$ T cells, are crucial in psoriasis pathogenesis¹⁵, with IL-17 playing a particularly important role. Cytokines are important initiators and regulators and are thus potential therapeutic targets. In the previous reports, monoclonal antibodies (mAbs) that target cytokine signaling were shown to successfully treat skin inflammation^{18, 66}. Dupilumab, which inhibits IL-4 and IL-13 dual signaling by targeting the IL-4 receptor alpha subunit, was also reported to have satisfactory efficacy and safety². Moreover, an anti-p40 monoclonal antibody, ustekinumab, which blocks IL-12 and IL-23, received FDA approval for treating plaque psoriasis¹⁵. In addition to extracellular cytokine-targeting drugs, JAK-STAT inhibitors such as tofacitinib have also been developed under the assumption that

targeting intracellular cytokine signaling is very attractive for a broad targeting approach⁶⁷. We hypothesized that TC-PTP is an important target for regulating various inflammatory cytokine signals and that TcR signaling could also be targeted to regulate the complex inflammatory response in dermatitis. Topical tacrolimus therapy is the current clinical treatment and has been shown to significantly improve the severity of AD, without affecting serum IgE levels or peripheral blood leukocytes.⁶⁸ These findings were presumably due to the blood concentrations of tacrolimus, which showed that the drug was rarely detected in most of the patients⁶⁹. Oral cyclosporine A therapy was used to treat the majority of patients with moderate to severe AD, some of whom had decreased serum IgE levels⁷⁰. We found that transcutaneous AP-rPTP treatment resulted in slightly decreased serum IgE levels (Fig E13A and B), suggesting that further investigations should investigate the systemic administration effects of AP-rPTP. We also aim to investigate AP-rPTP in future clinical trials.

In summary, here we demonstrated the technical feasibility of utilizing a novel transdermal delivery peptide (TDP), AP, in a transcutaneous protein therapeutic developed to treat inflammatory skin diseases. This peptide-based macromolecular delivery method can potentially improve topical drug delivery of small molecules, proteins, and antibodies across the skin tissue barrier. Transcutaneous application of AP-rPTP by the paper patch technique could modulate cytokine signaling and TcR signaling in a substrate-specific manner, making this treatment strategy highly attractive for treating inflammatory skin diseases such as atopic dermatitis and psoriasis.

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Figure Legends

FIG. 1. *In vitro* protein delivery efficiency into skin cells by the novel transdermal delivery peptide AP. **A**, Homology of the AP sequence. **B**, SDS-PAGE analysis of purified proteins. **C**, Dose-dependent and **D**, time-dependent protein delivery into HaCaT cells. Comparison of delivery efficiencies into HaCaT cells. Images of EGFP proteins in **F**, HaCaT cells and **G**, NIH3T3 cells acquired at $\times 100$ magnification (Scale bar, 200 μm) or with a confocal microscope acquired at $\times 600$ (Scale bar, 50 μm) magnification. White arrowheads indicate areas where EGFP signal colocalizes with Hoechst signal. **H**, Experimental scheme of mouse primary epidermal cell isolation. **I**, Gating strategy of flow cytometry analysis. **J**, Comparison of delivery efficiencies into isolated epidermal cells. Error bars indicate s.d. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

FIG. 2. *In vivo* protein delivery efficiency of AP into murine skin tissue. Biodistribution of AP-dTomato in the epidermis and dermis of GFP-transgenic mice at **A**, $\times 200$ (Scale bar, 100 μm) and **B**, $\times 600$ (Scale bar, 50 μm) magnification. Arrowheads indicate cells into which AP-dTomato was delivered. **C**, Comparison of *in vivo* transcutaneous delivery efficiencies. Multi-photon microscope imaging was performed. **D**, 3D lateral view (Scale bar, 50 μm); **E**, optical sections of images below the epidermis (40 μm) to a depth of 120 μm at intervals of 20 μm (Scale bar, 100 μm); **F**, Cumulative xyz images (40–120 μm) (Scale bar, 100 μm); and **G**, 3D cumulative image surface plot. **H**, Quantitative analysis of red fluorescence signals over the threshold in the dermis. Signals are represented as spheres, and **I**, Bar graph of the numbers of spheres. Quantitative analysis was performed by Imaris 8.2.0. Error bars indicate s.d. *** $P < 0.001$.

779 **FIG. 3. *In vitro* protein delivery efficiency of AP into human breast skin tissue samples**
780 **from healthy donors. A,** Experimental scheme. **B,** Image of protein delivery onto human
781 skin tissue. **C,** Bright field image of full thickness human skin tissue. **D,** Fluorescence
782 microscopy analysis. dTomato proteins were applied for 12 h onto human skin samples from
783 three different donors. Red fluorescence signal was observed at $\times 100$ magnification (Scale
784 bar, 200 μm). White arrowheads indicate areas where dTomato signal colocalizes with
785 Hoechst signal.

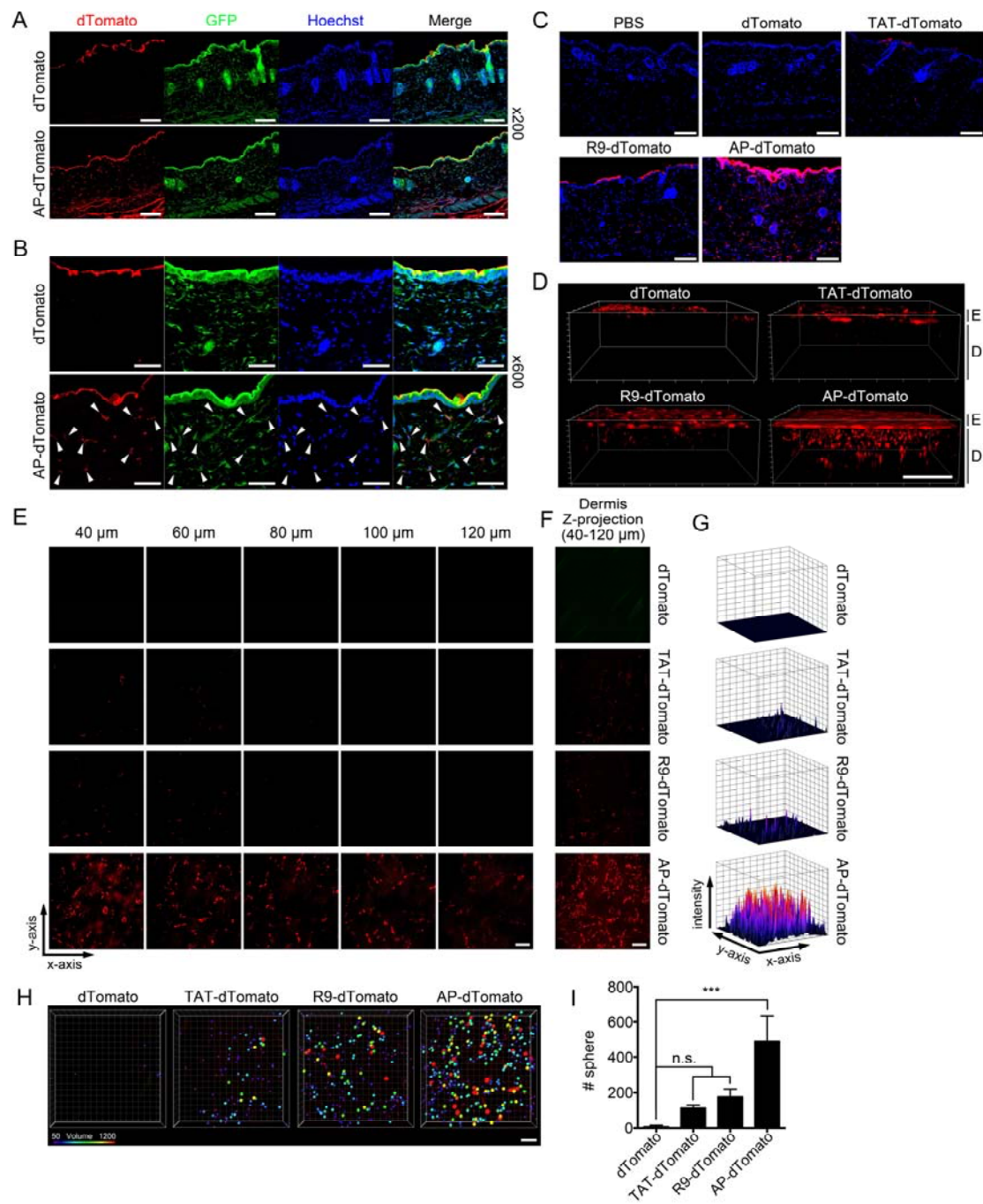
FIG. 4. Determining the mechanism of protein delivery into skin cells. The delivery mechanism of AP-EGFP was determined by treating HaCaT cells with **A**, heparin; **B**, methyl-beta-cyclodextrin (M β CD); or **C**, amiloride. **D**, Arginine length optimization of the AP sequence. **E**, Characterization of the AP sequence in AP by amino acid mutation. **F**, Effect of cysteine modification on transduction efficacy. Error bars indicate s.d. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

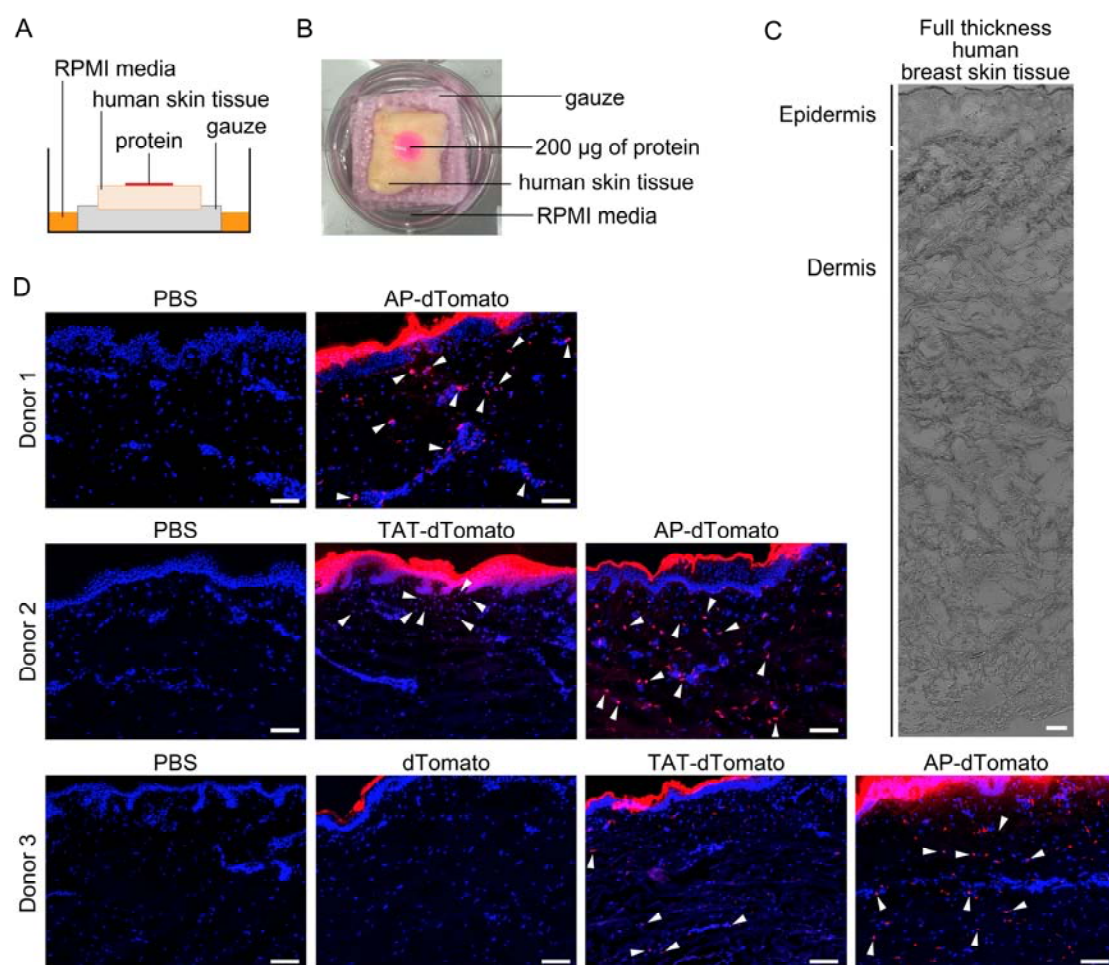
FIG. 5. Engineering protein tyrosine phosphatase into a novel immunomodulator. **A**, Schematic images of the structure of AP-rPTP. **B**, Analysis of purified protein. Protein was resolved by SDS-PAGE and subjected to immunoblot analysis with anti-His-tag antibodies. **C**, Computational 3D structure modeling of AP-rPTP. **D**, *In vitro* pNPP phosphatase activity assay of AP-rPTP. * $P < 0.001$. **E**, Dose-dependent and **F**, time-dependent delivery of AP-rPTP into HaCaT cells. **G**, Dephosphorylation of STAT by AP-rPTP in mouse splenocytes. **H**, Inhibition of CD4 T cell proliferation by AP-rPTP. **I**, Inhibition of cytokine production by AP-rPTP. Error bars indicate s.d. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

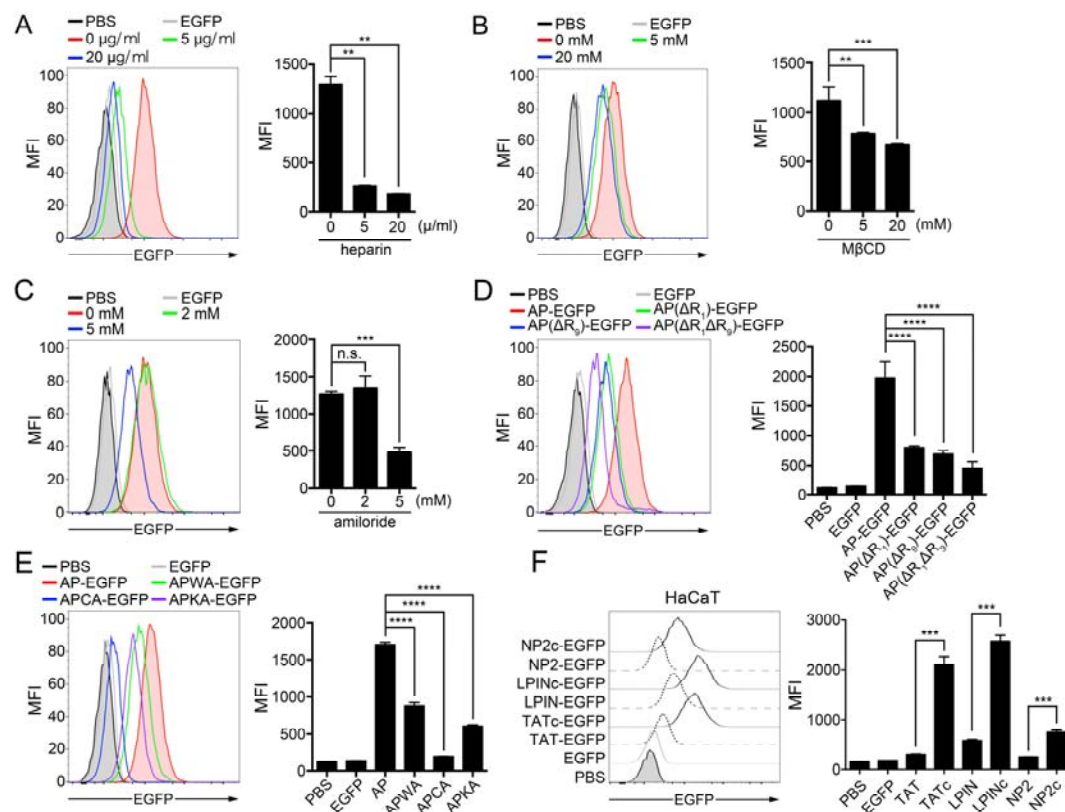
FIG. 6. Transdermal administration of AP-rPTP ameliorates allergic skin inflammation.

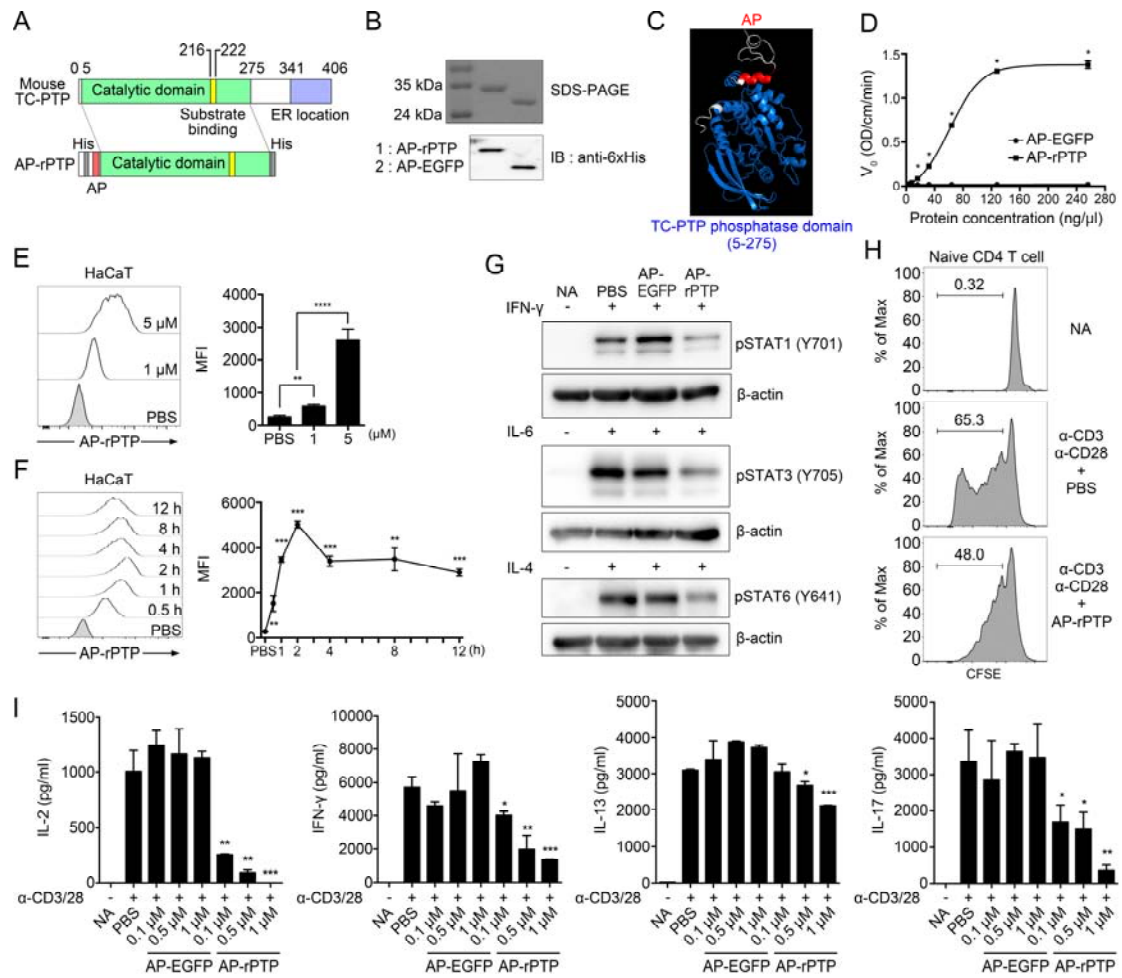
A, Scheme of the OXA-induced CHS model. **B**, Ear thicknesses of CHS mice measured every day. **C**, Ear punch biopsy weights. **D**, Representative gross images of the ear 24 h after challenge. **E**, Histological analysis of ear tissue (scale bar, 200 μ m). Quantification of **F**, cytokine and **G**, chemokine mRNA expression levels. **H**, Scheme of the OVA-induced atopic dermatitis-like model. **I**, Histological analysis of back skin (scale bar, 200 μ m). **J**, Total histology H&E scores. **K**, Quantification of epidermal thickness. Quantification of infiltrated **L**, neutrophils and **M**, eosinophils. Values are expressed per field. **N**, Toluidine blue staining and **O**, CD3 staining of mast cells and CD3 T cells, respectively. Quantification of infiltrated **P**, mast cells and **Q**, CD3 T cells. Values are expressed per field. **R**, Quantification of IL-13 cytokine mRNA expression levels in skin. **S**, TEWL measurements of back skin after the last OVA sensitization. Error bars indicate s.e.m. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

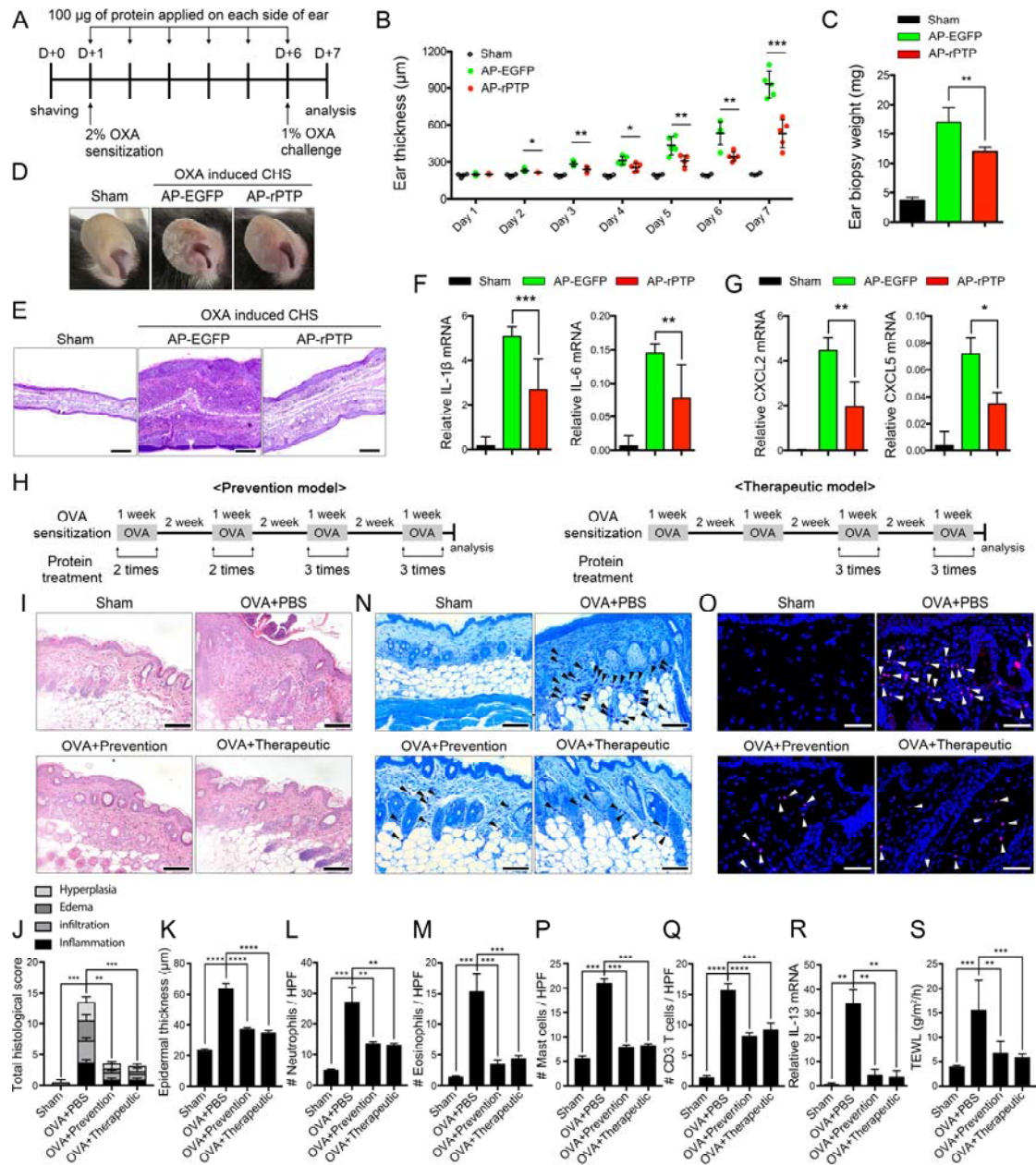
FIG. 7. Transdermal administration of AP-rPTP ameliorates psoriasis-like skin inflammation. **A**, Scheme of the imiquimod-induced psoriasis-like skin inflammation model. **B**, Ear thicknesses of mice with psoriasis-like skin inflammation. **C**, Histological analysis of ear tissue (scale bar, 200 μ m). **D**, Quantification of epidermal thickness. **E**, Quantification of infiltrated lymphocytes and neutrophils. Values are expressed per field. Quantification of **F**, cytokine and **G**, antimicrobial peptide mRNA expression levels in skin. **H**, Representative gross images of back skin 24 h after the last imiquimod challenge. **I**, Scoring of skin thickening, scaling, and erythema. **J**, Histological analysis of back skin (scale bar, 200 μ m). **K**, Quantification of epidermal thickness. Error bars indicate s.e.m. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

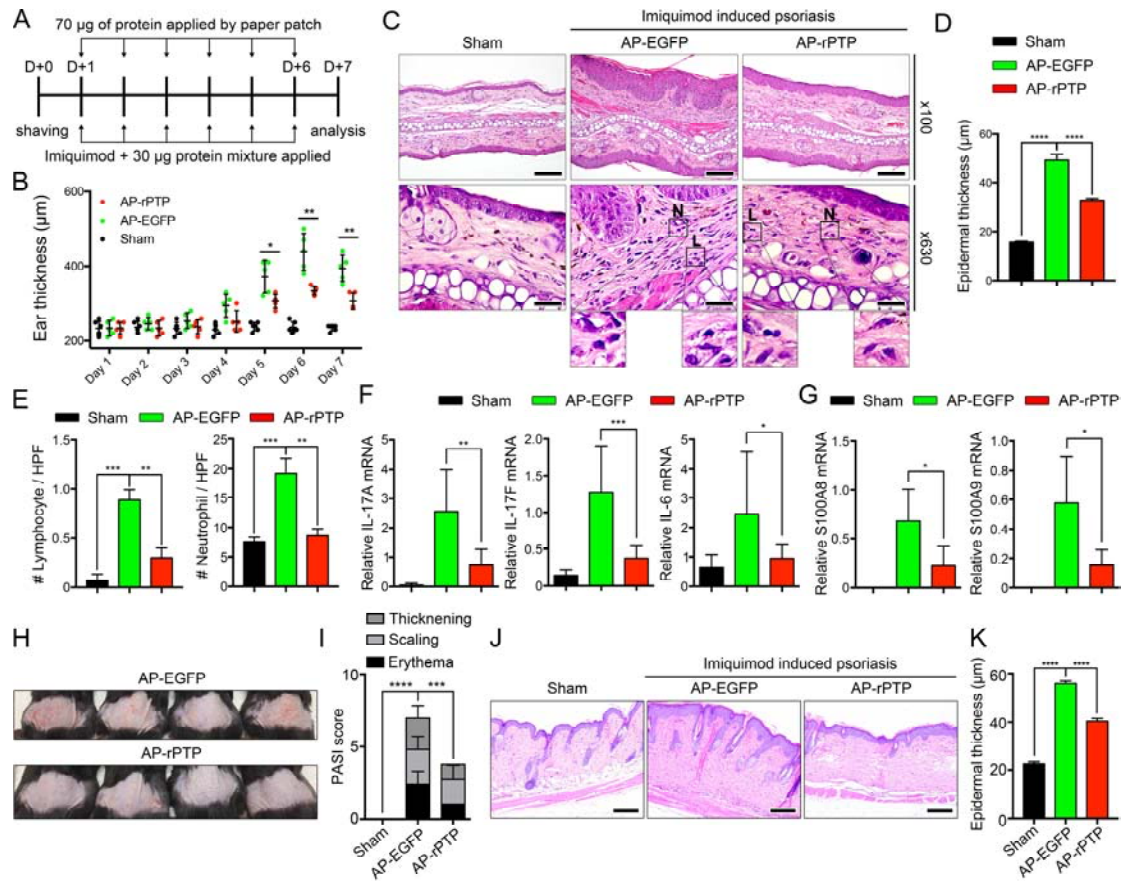


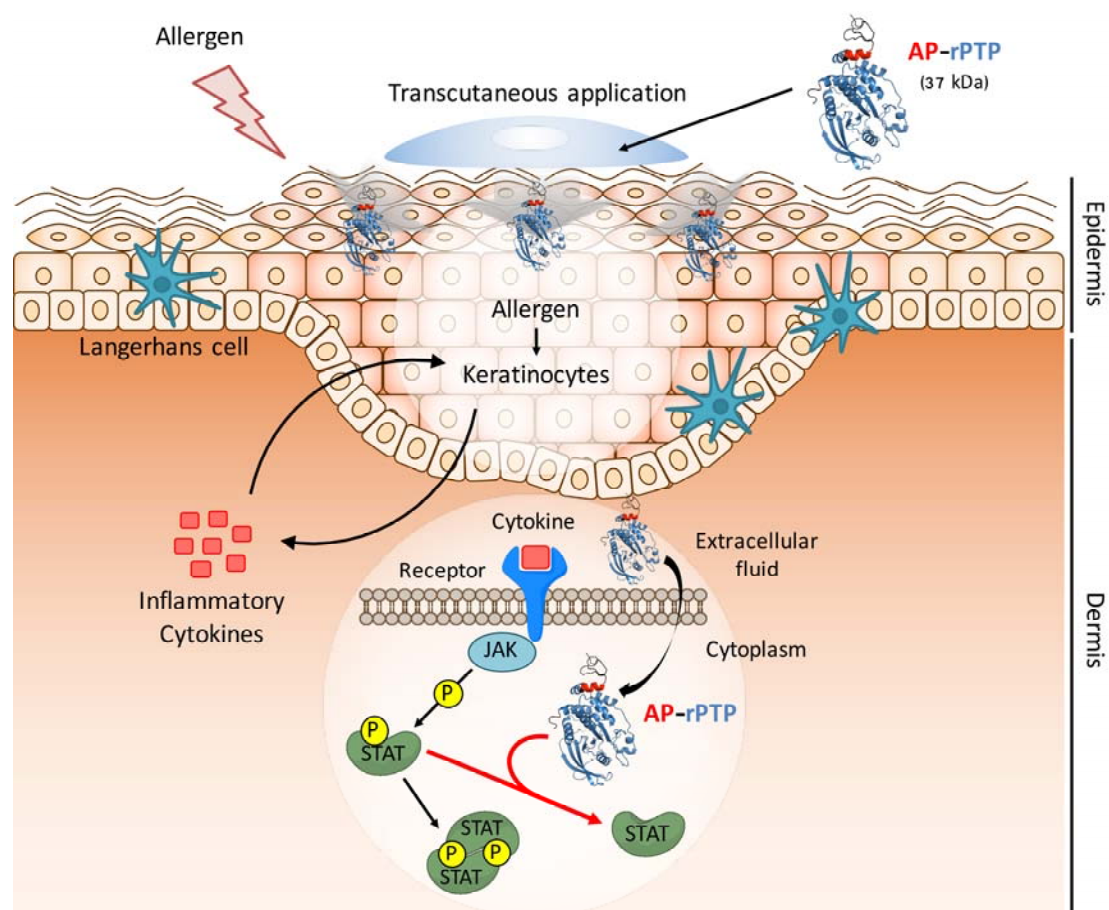












Online Repository**Protein tyrosine phosphatase conjugated with a novel transdermal delivery peptide, AP-rPTP alleviates both atopic dermatitis-like and psoriasis-like dermatitis****Methods**

Screen for novel transdermal delivery peptides. To screen for novel transdermal delivery peptides, we used the ScanProsite tool (<http://prosite.expasy.org/scanprosite/>). We scanned motif sequences including arginine, lysine, tryptophan, and cysteine against a UniProtKB protein database and filtered the results by *Homo sapiens* taxonomy. The access number of human astrotactin 1 protein in UniProt is O14525.

Purification of recombinant proteins. The purification protocol was modified from a previous method^{E1}. Recombinant proteins conjugated to AP and other control peptides were expressed and purified from *Escherichia coli*. Briefly, BL21 (DE3) pLysS and BL21 codon plus (DE3) cells were transformed with plasmids encoding recombinant fluorescent proteins and a TC-PTP phosphatase domain. Protein expression was induced with 0.2 mM isopropyl β -D-1-thiogalactopyranoside (IPTG; Gold Biotechnology, St. Louis, MO, USA), after which cells were cultured overnight at 20°C with shaking at 150 rpm. The cells were then harvested by centrifugation at 6,000 rpm for 15 min and resuspended in binding buffer (50 mM NaH₂PO₄, 300 mM NaCl, and 10 mM imidazole, pH 8.0). Cells were lysed with a Vibra-cell VCX-130 ultrasonic processor (Sonics and Materials Inc., Newton, CT, USA). The lysate was clarified by centrifugation, and the supernatant was sequentially filtered through 0.45 μ m and 0.22 μ m cellulose nitrate syringe filters (Sartorius Stedim Biotech GmbH, Goettingen,

Germany). 6x His-tagged target proteins were purified via Ni-NTA affinity chromatography (Qiagen, Chatsworth, CA, USA), after which the buffer was exchanged for SP binding buffer (10 mM sodium phosphate buffer and 10% glycerol; pH was adjusted as follows: $\text{pH} = \text{pI}$ of the recombinant protein - 0.7) using a PD-10 Sephadex G-25 column (GE Healthcare, Uppsala, Sweden). To increase protein purity, cation exchange chromatography was performed using SP Sepharose High Performance resin (GE Healthcare, Uppsala, Sweden). Finally, purified proteins were desalted using a PD-10 Sephadex G-25 column with PBS buffer containing 10% glycerol. Protein concentrations were measured using the Bradford assay and the purified proteins were stored at -80°C .

Cell culture. HaCaT (human keratinocytes) and NIH3T3 (mouse fibroblasts) cell lines were purchased from the American Type Culture Collection (ATCC) and cultured in DMEM (Cellgro; Mediatech, Washington DC, USA) containing 10% FBS and 1% penicillin/streptomycin (Hyclone, Logan, UT, USA). The Jurkat (human leukemia cells) cell line was purchased from ATCC and cultured in RPMI 1640 medium (Cellgro) containing 10% FBS (Cellgro) and 1% penicillin/streptomycin. All cells were cultured at 37°C in a 5% CO_2 incubator (Forma Scientific, Marietta, OH, USA).

Mice. C57BL/6 mice and BALB/c mice were purchased from Orient Bio (Daejeon, Korea). The mice were housed and bred in specific pathogen-free animal facilities at Hanyang University, Korea Advanced Institute of Science and Technology (KAIST), and Yonsei University. All animal procedures used in this study were approved by the Animal Care and Use Committees of Hanyang University, KAIST, and Yonsei University.

***In vitro* skin cell delivery.** HaCaT cells were seeded in 24-well plates (5×10^4 cells per well) and incubated at 37 °C overnight. The cells were then treated with recombinant proteins at the indicated concentrations (0.5, 1, 2, or 5 μ M for AP-EGFP delivery and 1 or 5 μ M for AP-rPTP delivery) or for different periods of time (0.5, 1, 2, 4, or 8 h for AP-EGFP delivery and 0.5, 1, 2, 4, 8, or 12 h for AP-rPTP delivery). The cells were washed once with PBS to eliminate uninternalized recombinant protein on the cell membrane, after which they were washed with 0.25% trypsin (Hyclone, Logan, UT, USA), incubated for 10 min at 37 °C, and washed with PBS. Intracellular AP-rPTP was immunostained with anti-His-tag antibodies (MBL, Nagoya, Japan) and the signal was amplified with Alexa Fluor 488-conjugated anti-mouse IgG antibodies (Molecular Probes Inc., Eugene, OR, USA). Intracellular fluorescence was analyzed by flow cytometry (FACS Canto II, BD Bioscience, San Jose, CA, USA) and the data were analyzed using FlowJo software version 10.0.7 (Tree Star, Inc., San Carlos, CA, USA).

Primary epidermal cells were obtained from the dorsal skin of C57BL/6 mice. To separate the epidermis and dermis, excised dorsal skin was incubated in 2.4 U/ml dispase II (Roche, Indianapolis, IN, USA) for 1 h at 37 °C and then washed with PBS. Next, the epidermis was scraped off using a scalpel. The epidermis was then digested with trypsin for 1 min and passed through a 0.70 μ m pore cell strainer (SPL Life Science, Pocheon, Korea). A total of 1.0×10^6 epidermal cells were incubated with the indicated recombinant fluorescent protein and the resultant intracellular fluorescence was analyzed by flow cytometry. The epidermal cells were stained with anti-mouse CD45-PerCP-Cy5.5, CD3-PE-Cy7, and CD11c-APC FACS antibodies purchased from BioLegend.

Intracellular delivery mechanism. HaCaT cells were seeded in 24-well plates (5×10^4 cells per well) and incubated at 37 °C overnight. The cells were pre-incubated with heparin (0, 20

or 50 µg/ml), MβCD (0, 5 or 20 mM), amiloride (0, 2 or 5 mM), or chlorpromazine (CPZ; 0, 5 or 10 µM) at 37°C for 30 min. The cells were then treated with 5 µM of recombinant protein for 1 h and washed as described above. Intracellular delivery efficiency was analyzed by flow cytometry.

Intracellular localization. HaCaT cells or NIH3T3 cells were seeded in 6-well plates (2 × 10⁵ cells/well) and incubated overnight. AP-fusion or other control fluorescent proteins (20 µM) were added for 2 h at 37 °C, after which the cells were washed three times with PBS and fixed with 4% paraformaldehyde for 10 min. Nuclei were stained with Hoechst 33342 (Invitrogen) for 15 min and the cells were washed three times with PBS. The cells were then mounted in Fluoroshield with DABCO mounting medium (Sigma-Aldrich, St. Louis, MO, USA). Intracellular fluorescence, which reflects the fusion protein localization, was analyzed using a confocal microscope (Nikon C2+ Instruments Inc.) or a fluorescence microscope (Leica DMI8, Leica Microsystems).

In vivo transcutaneous delivery. Hair was carefully removed from the dorsal skin of 7 to 9-week-old C57BL/6 mice or B6.129S1-Tg(CAG-EGFP)S1C2Tpo/KnwPeaJ mice using a clipper and depilatory cream. Mice were placed under anesthesia with zoletil (30 mg/kg; Virvac, Carros, France) and rompun (10 mg/kg; Bayer Korea, Ansan, Korea) (diluted in 180 µl PBS). The dorsal skin was then tape-stripped 10 times. A total of 100 µg of recombinant dTomato protein was applied onto Whatman paper (Whatman, Clifton, NJ, USA) and occluded with transparent adhesive film (Tegaderm, 3M, St. Paul, MN, USA). Before harvesting the tissue for analysis, the skin was cleaned with 70% ethanol to remove residual protein. The tissue was harvested and fixed with 4% paraformaldehyde for 1 h at room temperature and then dehydrated with 30% sucrose until deposition. Skin tissue samples were

frozen using OCT compound (Leica, Germany). The frozen blocks were vertically sectioned using a cryostat (Thermo Scientific, Logan, UT, USA). Slices were obtained with a thickness of 10 μ m for fluorescence microscopy or 20 μ m for confocal microscopy. Cryosections were stained with Hoechst 33342 dye for nuclei visualization and enveloped with Fluoroshield and DABCO mounting medium. The slides were analyzed on a fluorescence microscope (Leica DMi8, Leica Microsystems) and a confocal microscope (Nikon Instruments Inc.). Following acquisition, images were analyzed using ImageJ version 2.0.0 (National Institute of Health, Bethesda, MD, USA).

Multi-photon microscopy of live animals. Recombinant proteins were transcutaneously applied as described above to the dorsal skin of C57BL/6 mice (7 to 9-week-old, males and females). The mice were then anesthetized via a mixture of zoletil (30 mg/kg) with rompun (10 mg/kg) to stabilize the animals. Body temperature (37-38 °C) was maintained using a homoeothermic heating pad system controlled by a rectal probe. The animal's physiological health was closely monitored throughout the entire procedure. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Sungkyunkwan University. A chamber plate (CP-1, Narishige Inc., Tokyo, Japan) with a 5-mm observation hole was placed on the skin. The observation window was sealed with cyanoacrylate glue, after which the animals were placed in a chamber plate-fixing apparatus (MAG-1, Narishige Inc., Tokyo, Japan) for multi-photon imaging (TCS SP8 MP, Leica Microsystems CMS GmbH, Mannheim, Germany). Excitation was performed using a 920-nm Ti:Sapphire laser (Chameleon Vision II, Coherent Inc., Santa Clara, CA) and the emitted fluorescent signal was detected on a hybrid detector through a 585/40 band-pass filter cube. Images were acquired using a 25x water-immersion objective lens (N.A. 0.95) with a 0.5 μ m step size and 512x512 pixels. The imaging depth was approximately 120 μ m from the skin

surface. Following acquisition, images were analyzed using LAS AF 3.2.0 (Leica Microsystems CMS GmbH) software and Imaris 8.2.0 software.

***In vitro* transcutaneous delivery in human skin tissue.** Using depilatory cream, hair was removed from human breast skin tissue samples obtained from three different donors. After thorough washing with PBS, tape stripping was performed 15 times to remove the stratum corneum. Prepared human skin samples (2 cm X 2 cm) were excised and placed on pieces of gauze soaked with RPMI 1640 (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS and 1% penicillin/streptomycin. The gauze pieces were placed in a 6-well plate. Next, 200 µg of protein was applied on Whatman paper disks (8 mm in diameter) and the lid was closed to generate a humidified environment. After a 12 h incubation at 37 °C, the Whatman paper was removed and the skin surface was washed with 70 % ethanol to remove residual protein. The human skin tissue samples were frozen using OCT compound (Leica, Germany). The frozen blocks were vertically sectioned using a cryostat (Thermo Scientific, Logan, UT, USA). Slices with a thickness of 10 µm were obtained. Cryosections were stained with DAPI dye for nuclei visualization and enveloped with Fluoroshield and DABCO mounting medium. The slides were analyzed on a fluorescence microscope (Zeiss Axio Observer, Carl Zeiss Inc.). Following acquisition, images were analyzed using ImageJ, version 2.0.0 (National Institutes of Health, Bethesda, MD, USA).

Phosphatase activity assay. The phosphatase activity of each purified rPTP protein was measured using the pNPP (*para*-nitrophenyl phosphate) phosphatase assay (BioAssay Systems, Hayward, CA, USA) following the manufacturer's protocol. Briefly, purified AP-EGFP and AP-rPTP were serially diluted with PBS and incubated with an equal volume of

pNPP substrate solution (50 μ l) for 10 min. Absorbance was measured at 405 nm using a spectrophotometer (Thermo Fisher Scientific).

Cytokine signaling and immunoblot analysis. Mouse splenocytes from 6 to 8-week-old C57BL/6 mice were incubated with PBS, AP-EGFP, or AP-rPTP for 1 h at 37 °C. Next, splenocytes were activated at 37 °C with the following cytokines: recombinant mouse IFN- γ (10 ng/ml; BD) for 60 min, IL-4 (20 ng/ml; BD) for 15 min, and IL-6 (30 ng/ml; BD) for 30 min. The cells were then washed with PBS and lysed with RIPA buffer (Cell Signaling, Beverly, MA, USA) containing 1 mM NaF, 1 mM PMSF, and the Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA) for 30 min on ice. Immunoblotting was performed using PVDF membranes (Bio-Rad) probed with the following primary antibodies: anti-phospho-Stat1 (Tyr701), anti-phospho-Stat3 (Tyr705), and anti-phospho-Stat6 (Tyr641) (all rabbit mAbs from Cell Signaling), and a mouse mAb against β -actin (Santa Cruz Biotechnology, CA, USA).

T cell activation and proliferation. Mouse splenocytes isolated from 6 to 8-week-old C57BL/6 were stimulated in a 96-well plate for three days at 37 °C with plate-bound anti-CD3 (2 μ g/ml; BD Pharmingen) and anti-CD28 (2 μ g/ml; BD Pharmingen) antibodies in the presence of AP-EGFP and AP-rPTP. The culture supernatant levels of the cytokines IFN- γ (BioLegend), IL-2 (BioLegend), IL-13 (Ebioscience), and IL-17 (Ebioscience) were analyzed by ELISA.

Naïve CD4⁺CD62L⁺CD44⁺CD25⁻ T cells were sorted from 6 to 8-week-old female C57BL/6 mice using a FACS Aria III instrument (BD). The FACS-sorted naïve CD4⁺ T cells were labeled with 1.25 μ M CFSE (Invitrogen, Carlsbad, CA, USA) for 10 min at 37 °C. Labeled cells were then washed twice and resuspended in RPMI 1640 medium supplemented

with 10% FBS, 1% penicillin/streptomycin, 2 mM l-glutamine, 25 mM HEPES, and 50 μ M beta-mercaptoethanol. The cells were stimulated with plate-bound anti-CD3 (2 μ g/ml; BD Pharmingen) and anti-CD28 (2 μ g/ml; BD Pharmingen) antibodies for 3 days. T cell proliferation was analyzed by flow cytometry based on CFSE peak division.

OXA-induced contact hypersensitivity mouse model. The OXA-induced contact hypersensitivity model was generated as previously described^{E2}, but with some modifications. Briefly, OXA (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one; Sigma-Aldrich, St. Louis, MO, USA) in AOO (acetone:olive oil = 4:1) was applied on the ears of 7 to 8-week-old female C57BL/6 mice to induce contact hypersensitivity. On day 0, depilatory cream was applied for 2 min, after which the cream was removed with distilled water. On day 1, both sides of the ears were sensitized with 10 μ l of 2% OXA. On day 6, both sides of the ear were challenged with 10 μ l of 1% OXA. In the back skin model, 30 μ l of OXA was used for both sensitization and challenge. After 24 h of challenge, the mice were sacrificed and tissue inflammation was analyzed. From day 1 to day 6, 100 μ g of protein was applied on each side of the ear for 1 h by the paper patch method. For the back skin model, 500 μ g of each protein was applied on a patch of sterile gauze and then secured to the skin with a transparent bio-occlusive dressing (Tegaderm) for 4 h. Ear thickness was measured before protein administration each day using a digital micrometer (Mitutoyo, Kawasaki, Japan). All sensitization and challenge treatments were performed under light anesthesia with isoflurane and all protein application was performed under deep anesthesia with zoletil (30 mg/kg) and rompun (10 mg/kg) (diluted in 180 μ l PBS). For histological analysis, 6-mm biopsy punches were excised from the ears and fixed with 4% paraformaldehyde. For mRNA analysis, the remaining ear tissue was stored at -80 °C in TRI Reagent RT (Molecular Research Center, Inc., Cincinnati, OH, USA).

OVA-induced chronic dermatitis mouse model. The OVA (ovalbumin)-induced chronic atopic dermatitis-like mouse model was generated as previously described^{E3}. Briefly, female BALB/c mice were used at 7-8 weeks of age. For sensitization, 100 µg of OVA was applied to sterile gauze on the dorsal skin of the mice for one week and then removed. This procedure was repeated four times at 2-week intervals. For the prevention scheme, 100 µg of AP-rPTP was coadministered with OVA during all four sensitizations. For the therapeutic scheme, AP-rPTP was coadministered with OVA only in the last two sensitizations. Sensitization and protein administration were performed under light anesthesia with isoflurane. After the fourth sensitization, the mice were sacrificed and tissue inflammation was analyzed via H&E, toluidine blue, and immunofluorescence staining. mRNA expression levels were also analyzed by quantitative PCR. Epidermal thicknesses were measured by randomly drawing at least 25 line segments from the stratum corneum to the epidermal-dermal junction for each mouse using a bright field microscope. The numbers of infiltrated immune cells, including neutrophils, eosinophils, mast cells, and CD3 T cells, were counted in five randomly selected fields (magnification x400 for neutrophils, eosinophils and CD3 T cells and magnification x200 for mast cells) for each mouse. Dermatitis severity was evaluated by scoring epidermal hyperplasia, edema, immune cell infiltration, and inflammation on a scale ranging from 0 to 4 (no symptoms, 0; mild, 1; moderate, 2; severe, 3; very severe, 4).

Imiquimod-induced psoriasis-like dermatitis mouse model. The imiquimod-induced psoriasis-like skin inflammation model was generated as described^{E4}. Briefly, male C57BL/6 mice were used at 7-8 weeks of age. For 6 consecutive days, 20 mg of Aldara cream (3M Pharmaceuticals, Leicestershire, United Kingdom) was applied on the skin of each ear; alternatively, 40 mg was applied on the back skin. Bilateral ear thickness was measured every

day before protein treatment using a micrometer (Mitutoyo, Kawasaki, Japan). The paper patch method was used to apply 70 µg of each protein onto the ventral and dorsal ears for 1 h, after which 20 mg of Aldara cream and 30 µg of protein (1:1 ratio) were applied on both ears daily. For the back skin model, 500 µg of each protein was applied on a patch of sterile gauze and then secured to the skin for overnight using a transparent bio-occlusive dressing (Tegaderm). Protein application was performed under deep anesthesia with zoletil (30 mg/kg) and rompun (10 mg/kg) (diluted in 180 µl PBS) for the paper patch method and light anesthesia with isoflurane for the Aldara cream mixture. On day 7, mice were sacrificed and tissue inflammation was analyzed with H&E staining. Additionally, mRNA expression levels were measured by quantitative PCR. The severity of inflammation was evaluated by scoring erythema, scaling, and thickening on a scale ranging from 0 to 4 (no symptoms, 0; mild, 1; moderate, 2; severe, 3; very severe, 4).

RNA extraction and quantitative PCR. RNA was extracted from ear tissue with TRI Reagent RT (Molecular Research Center, Inc.) according to the manufacturer's protocol. The isolated RNA was quantified with a UV spectrophotometer (CellTAGen, Inc., Seoul, Korea). cDNA was synthesized with a ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). Quantitative RT-PCR was performed with iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) using a Bio-Rad CFX Connect real-time PCR detection system. Expression was normalized to β -actin or GAPDH, as indicated in the figure legends. The following primers were used: IL-6 (forward) 5'-AGGATACCACTCCCAACAGACCT-3' and (reverse) 5'-CAAGTGCATCATCGTTGTTTCATAC-3'; IL-13 (forward) 5'-CCTGGCTCTTGCTTGCCTT-3' and (reverse) 5'-GGTCTTGTGTGATGTTGCTC-3'; CXCL2 (forward) 5'-TCCAGAGCTTGAGTGTGACGC-3' and (reverse) 5'-TGGATGATTTTCTGAACCAGG-3'; CXCL5 (forward) 5'-

GCCCTACGGTGGAAGTCATA-3' and (reverse) 5'-GTGCATTCCGCTTAGCTTTC-3';
and IL-1 β . Primer sequences were obtained from PrimerBank
(<http://pga.mgh.harvard.edu/primerbank>). In the psoriasis mouse model, mRNA expression
levels were detected with a TaqMan gene expression assay (Applied Biosystems, Warrington,
UK). IL-17A, IL-17F, IL-6, S100A8, and S100A9 mRNA levels were normalized to that of
GAPDH.

Skin tissue histology. For H&E and toluidine staining, mouse ear and back skin samples
were fixed in 4% paraformaldehyde overnight and embedded in paraffin. The tissue blocks
were sectioned and stained with hematoxylin, eosin, and toluidine blue. For
immunofluorescence staining, sections were rehydrated and antigen retrieval was then
performed using sodium citrate buffer (10 mM sodium citrate). Nonspecific binding sites on
back skin sections were blocked with 5% BSA for 2 h at room temperature, after which the
sections were incubated overnight at 4 °C with anti-mouse CD3 antibodies, followed by
Hoechst staining for 10 minutes. Stained samples were enveloped with Fluoroshield and
DABCO mounting medium. The slides were analyzed on an inverted microscope (Leica
DMi8, Leica Microsystems). Following acquisition, images were analyzed using ImageJ
version 2.0.0 (National Institute of Health, Bethesda, MD, USA).

Statistics. Data were analyzed by two-tailed Student's t-tests, one-way ANOVA, or two-way
ANOVA using GraphPad Prism, version 6.0 (GraphPad Software, San Diego, CA, USA). P
values < 0.05 were considered significant.

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Figure Legends

Figure E1. Emission wavelengths of recombinant fluorescent proteins. Consistent emission wavelengths of purified recombinant EGFP with or without the AP, R9, or TAT sequence. Emission wavelengths were measured with a fluorometer.

Figure E2. *In vitro* protein delivery efficiency into fibroblasts (NIH3T3 cells). NIH3T3 cells were incubated with 5 μ M of each protein for 1 h. Intracellular fluorescence was analyzed by flow cytometry. Left, histogram of MFI; right, bar graph of MFI. Error bars indicate s.d. ****P<0.0001.

Figure E3. Comparative microscopic analysis of AP-EGFP, R9-EGFP, and TAT-EGFP delivery efficiency into skin cells. **A**, Keratinocytes (HaCaT cells) and **B**, fibroblasts (NIH3T3 cells) were incubated with EGFP, TAT-EGFP, R9-EGFP, or AP-EGFP (20 μ M) for 1 h. Intracellular fluorescence was then visualized at $\times 100$ magnification using a fluorescence microscope (Scale bar, 200 μ m).

Figure E4. *In vitro* protein delivery efficiency into primary epidermal cells. Isolated mouse primary epidermal cells were incubated with 5 μ M of each protein for 1 h and analyzed by flow cytometry. The delivery efficiency was presented as dot plot.

Figure E5. Emission wavelengths of recombinant fluorescent proteins. Consistent emission wavelengths of purified recombinant dTomato with or without the AP, R9, or TAT sequence. Emission wavelengths were measured with a fluorometer.

314

315 **Figure E6. Protein delivery mechanism of AP into keratinocytes.** **A**, HaCaT cells were
 316 pre-incubated with chlorpromazine (CPZ), after which they were further incubated with 5 μ M
 317 of EGFP or AP-EGFP for 1 h. Left, histogram of MFI; right, bar graph of MFI. * $P < 0.05$. **B**,
 318 HaCaT cells were incubated with 5 μ M of EGFP, TAT-EGFP, R9-EGFP, or AP-EGFP at each
 319 temperature for 1 h. Left, histogram of MFI; right, bar graph of MFI. * $P < 0.05$; ** $P < 0.01$. **C**,
 320 HaCaT cells were incubated with 5 μ M of indicated proteins at 37 °C for 1 h in medium
 321 containing 0% or 10% serum. Left, histogram of MFI; right, bar graph of MFI. All data were
 322 analyzed by flow cytometry. Error bars indicate s.d. * $P < 0.05$; ** $P < 0.01$.

323

324 **Figure E7. Transdermal administration of AP-rPTP ameliorates immune cell**
 325 **infiltration in the oxazolone-induced CHS model.** **A**, High magnification histology images
 326 from each group. **B**, Quantification of immune cell infiltration. N indicates neutrophils and E
 327 indicates eosinophils.

328

329 **Figure E8. Transdermal administration of AP-rPTP ameliorates allergic skin**
 330 **inflammation in the oxazolone-induced CHS back skin model.** **A**, Histological analysis of
 331 back skin. **B**, Scoring of skin thickening, scaling, and erythema.

332

333 **Figure E9. Transdermal administration of AP-rPTP ameliorates inflammatory**
 334 **responses in the ovalbumin-induced atopic dermatitis-like model.** **A**, High magnification
 335 ($\times 400$) images of H&E-stained tissue samples. N indicates neutrophils and E indicates
 336 eosinophils. **B**, Total IgE and OVA-specific IgE levels, as measured by ELISA.

337

Figure E10. Transdermal administration of AP-rPTP ameliorates inflammatory responses in the ovalbumin-induced atopic dermatitis-like model. **A**, High magnification ($\times 400$) images of H&E-stained tissue samples. N indicates neutrophils and E indicates eosinophils.

Figure E11. Comparative flow cytometry analysis of AP-dTomato delivery efficiency in immune and skin cells with TDP peptides screened by phage display. **A**, Jurkat cells, **B**, HaCaT cells and **C**, NIH3T3 cells were incubated with 5 μ M of dTomato, AP-dTomato, MTD-1067 (Patent from Procell Therapeutics Inc.), or S3-2 (published peptide from LG Household & Health Care)-dTomato for 1 h. Intracellular fluorescence was analyzed by flow cytometry. Left, histogram of MFI; right, bar graph of MFI. Error bars indicate s.d. ****P<0.0001.

Figure E12. In vivo protein delivery efficiency by AP into murine skin tissue depends on tape stripping or inflammation. **A**, Fluorescence microscopy analysis of delivery on intact skin ($\times 50$ magnification). Intact skin indicates skin from which hair was removed (i.e. only depilatory cream was applied). Tape stripped skin indicates skin from which hair and the stratum corneum were both removed (i.e. depilatory cream application followed by tape stripping). **B**, Fluorescence microscopy analysis of delivery on inflammatory skin. Samples were analyzed at $\times 100$ magnification using a fluorescence microscope.

Figure E13. Transdermal administration of AP-rPTP slightly ameliorates serum IgE level in the ovalbumin-induced atopic dermatitis-like model. **A**, Total IgE and **B**, OVA-specific IgE levels were measured by ELISA. Error bars indicate s.d. Statistical analysis was performed by one-way ANOVA. *P<0.05.

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