

Intranasal delivery of the cytoplasmic domain of CTLA-4 using a novel protein transduction domain prevents allergic inflammation

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CTLA-4 is a negative regulator of T-cell activation, and its inhibitory effects can be accomplished either by competition with CD28 or by transmitting negative signals through its intracellular domain. To utilize the cytoplasmic domain of CTLA-4 to suppress allergic inflammation, we fused it to a novel protein-transduction domain in the human transcriptional factor Hph-1. Transduction efficiency was verified *in vitro* and *in vivo* after ocular, intranasal and intradermal administration. After transduction into T cells, the Hph-1-ctCTLA-4 fusion protein inhibited the production of interleukin (IL)-2, and downregulated CD69 and CD25. Intranasal administration of Hph-1-ctCTLA-4 resulted in markedly reduced infiltration of inflammatory cells, secretion of T helper type 2 (T_H2) cytokines, serum IgE levels and airway hyper-responsiveness in a mouse model of allergic airway inflammation. These results indicated that Hph-1-ctCTLA-4 constitutes an effective immunosuppressive protein drug for potential use in the treatment of allergic asthma, via nasal administration.

Asthma is a chronic lung disease in which inhalation and exhalation are obstructed by the production of excess mucus and the swelling of the airway membranes, giving rise to coughing and wheezing¹. Studies indicate that activation of CD4⁺ T helper lymphocytes of the T_H2 subset is important to trigger allergic asthma through the production of cytokines including IL-4, IL-5 and IL-13, which stimulate and recruit inflammatory cells²⁻⁴. CTLA-4 is an activation-induced surface molecule on T cells and is essential for the negative regulation of T-cell activation. It binds to B7-1 or B7-2 on antigen-presenting cells (APCs) with an affinity that is 10- to 20-fold higher than that of CD28. The cytoplasmic domain of CTLA-4 is known to interact with the phosphatases SHP-1 or SHP-2, resulting in dephosphorylation of CD3-TCR chains, ERK and JNK, thereby inhibiting T-cell

activation⁵⁻¹¹. The cytoplasmic domain of CTLA-4 containing a tyrosine motif is 100% conserved among different species, suggesting that this domain is important for negative regulation of T-cell activation by CTLA-4 (refs. 12,13).

Small protein domains, commonly referred to as protein transduction domains (PTDs), have been developed for the delivery of therapeutic proteins into eukaryotic cells¹⁴⁻¹⁷. Although fusion proteins composed of PTDs and various intracellular signaling mediators have elicited therapeutic effects in various *in vitro* and *in vivo* disease models, the lack of tissue specificity of PTDs has limited therapeutic application. Local administration of the therapeutic fusion proteins might ameliorate this concern.

We identified a novel cell-permeable PTD YARVRRRGPRR from the human transcriptional factor Hph-1. To determine the protein transduction efficiency of this domain, we assessed its ability to mediate the transduction of β -galactosidase into various cell lines, including HeLa, HepG2, HaCat and NIH3T3. First, we generated DNA constructs and expressed and purified Hph-1- β -galactosidase (Hph-1- β -gal) or β -gal only (Fig. 1a). Hph-1- β -gal (0.5–10 μ M) was readily transduced into the cultured cells, first detected at 15 min, and the maximum intracellular concentration was reached within less than 60 min (Fig. 1b). Nearly 100% of the cells became positive for β -gal enzyme activity after 1 h of incubation (Fig. 1c).

To monitor the intracellular location of Hph-1-PTD in cells, we incubated HeLa cells with 10 μ M Hph-1-enhanced green fluorescent protein (EGFP) fusion protein for 30 min (Fig. 1d). Hph-1-EGFP accumulated in the nucleus within a few minutes after incubation, although the fusion protein could also be found in the cytoplasm of the treated cells.

We next injected mice intraperitoneally with Hph-1- β -gal or β -gal only, and isolated liver, heart, kidney, lung, spleen and brain 4–6 h after injection (Fig. 1e). All of the tissues isolated from the mice injected with Hph-1- β -gal, but not β -gal only, showed strong

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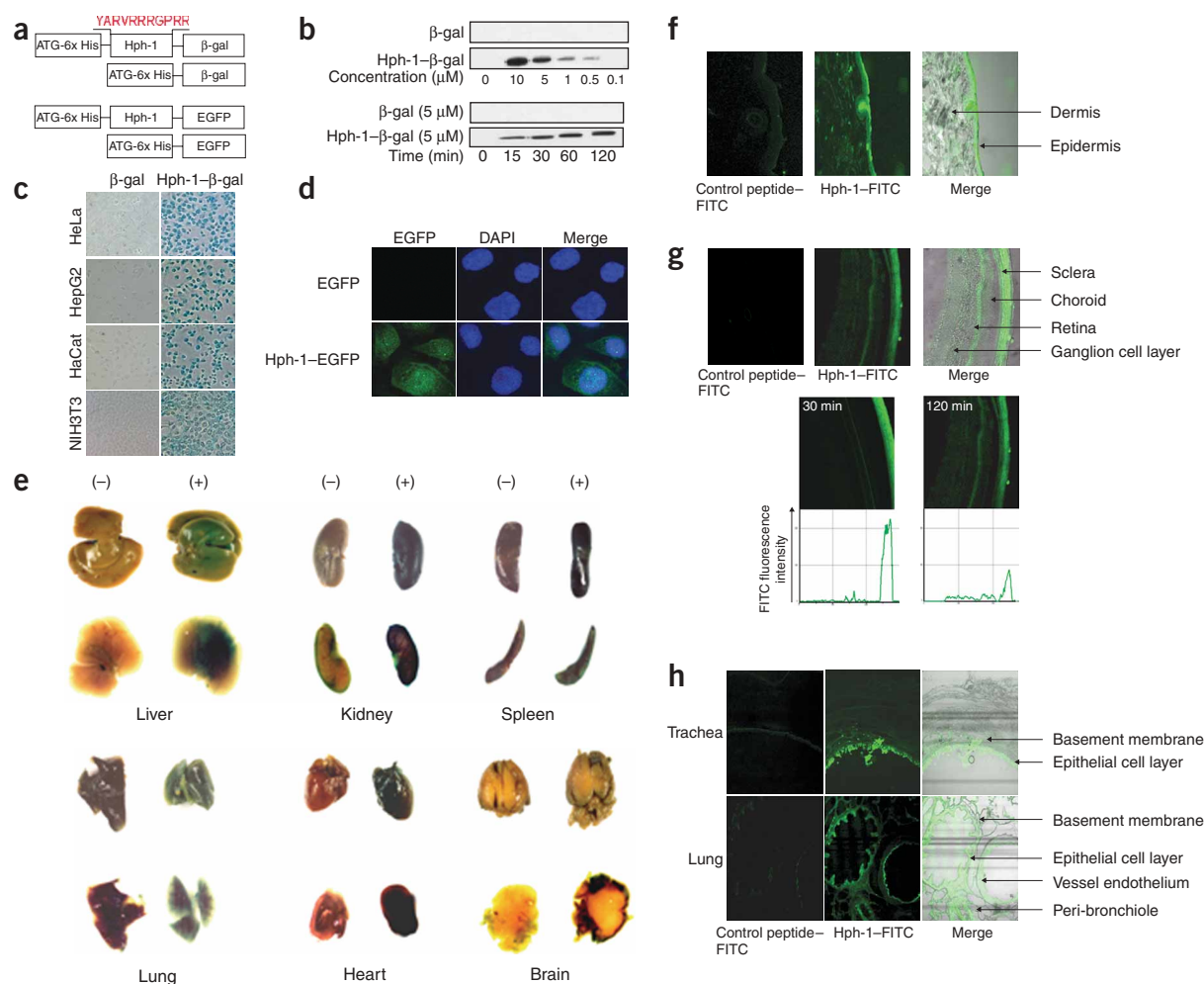


Figure 1 Evaluation of Hph-1 as a novel protein-transduction domain. **(a)** Structure of the Hph-1-PTD conjugated fusion proteins. **(b)** Transduction activity and kinetics of the Hph-1-β-gal analyzed by western blotting in Jurkat T cells. **(c)** Transduction efficiency of Hph-1 in several cell lines: HeLa, HepG2, HaCat and NIH3T3. After 1-h incubation with Hph-1-β-gal, cells were fixed and stained with X-gal solution. **(d)** Intracellular localization of Hph-1-EGFP. After 10 μM of Hph-1-EGFP or control EGFP, protein was incubated with HaCat cells for 1 h and intracellular fluorescence was analyzed by confocal microscopy. **(e)** *In vivo* transduction efficiency of Hph-1-PTD. We injected mice intraperitoneally with 5 mg Hph-1-β-gal or control β-gal. Liver, kidney, spleen, lung, heart and brain were fixed, and the whole organs (top row) and thin sections (bottom row) of each were analyzed for the β-gal activity with addition of X-gal solution. **(f)** Transduction of Hph-1-FITC into the epidermis as well as dermis through the skin. After Hph-1-FITC was applied to the stripped skin of hairless mice for 2 h, skin samples were analyzed by confocal microscopy. **(g)** Delivery of Hph-1-FITC into retina and ganglion cell layer through sclera. After Hph-1-FITC was dropped on the eye for 30 or 120 min, eye samples were analyzed by confocal microscopy. Monitoring plot of FITC fluorescence intensity across eye samples after ocular administration of Hph-1-FITC for 30 or 120 min. **(h)** Transduction of Hph-1-FITC through the nasal route. After Hph-1-FITC was injected by nasal instillation for 30 min, fluorescence in the left main lung and trachea was analyzed by confocal microscopy.

and uniform β-gal activity, indicating that Hph-1-PTD can deliver a large protein into various organs and even across the blood-brain barrier in a concentration-dependent manner (**Supplementary Fig. 1** online).

To test the potential of Hph-1-PTD for therapy of local diseases, we administered fluorescein isothiocyanate (FITC)-labeled Hph-1-PTD through skin, eye or nasal airway. After topical application of Hph-1-FITC in ointment to the skin of hairless mice (**Fig. 1f**), we observed strong FITC fluorescence in the dermis as well as the epidermis. After ocular administration of Hph-1-FITC in PBS, Hph-1-FITC penetrated into the retina and the ganglion cell layer through the sclera (**Fig. 1g**). After delivery of Hph-1-FITC into the trachea and lung by intratracheal instillation, we detected fluorescence in microbronchi, in the endothelial area of the vessel in the lung, and in the basement membrane of the bronchus (**Fig. 1h**).

To test the therapeutic potential of Hph-1-PTDs for local suppression of the immune response in an experimental asthma model, we constructed a fusion protein consisting of Hph-1 and the cytoplasmic domain of CTLA-4 (Hph-1-ctCTLA-4) (**Fig. 2a**). We also constructed control proteins consisting of ctCTLA-4 only (no Hph-1) and Hph-1-ctCTLA-4YF, in which two tyrosines essential for the negative function of CTLA-4 were mutated into phenylalanines. The transduction of Hph-1-ctCTLA-4 into Jurkat T cells was concentration dependent, and maximal levels of intracellular Hph-1-ctCTLA-4 were reached at 60 min after incubation (**Fig. 2b**). To examine whether the delivered Hph-1-ctCTLA-4 could inhibit T-cell activation, we treated Jurkat T cells with Hph-1-ctCTLA-4 and stimulated them with CD28-specific and plate-bound CD3-specific monoclonal antibodies. Intracellular Hph-1-ctCTLA-4 substantially inhibited induction of CD69 and CD25 (**Fig. 2c**). The intracellular presence of Hph-1-ctCTLA-4 in

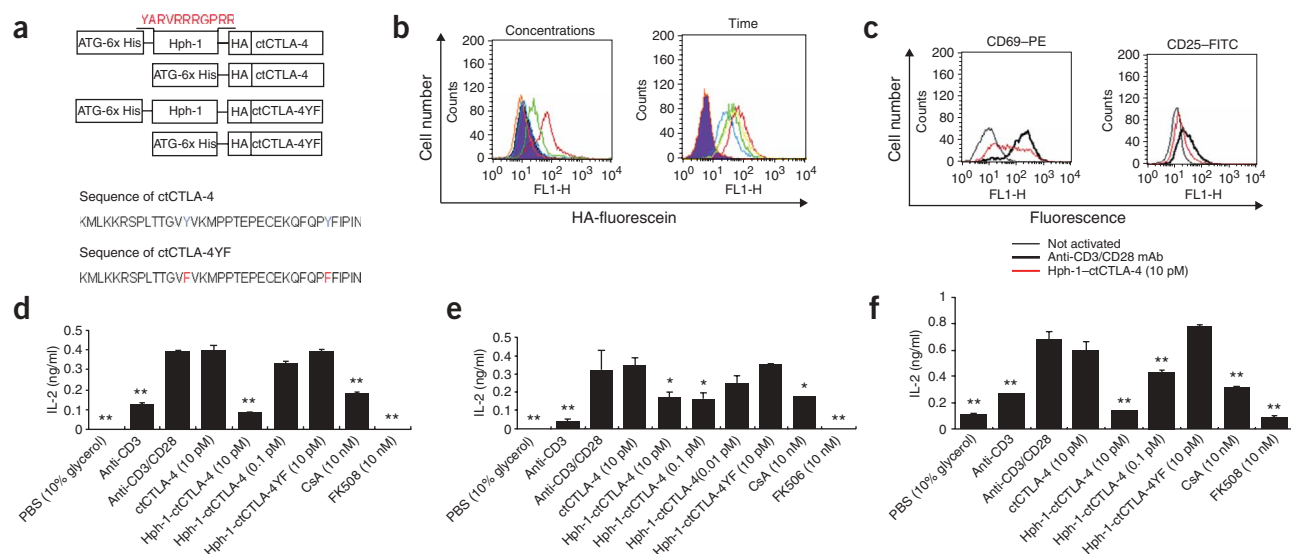


Figure 2 Inhibitory effect of Hph-1-ctCTLA-4 on T-cell activation. **(a)** Structure of Hph-1-ctCTLA-4 and Hph-1-ctCTLA-4YF. **(b)** Transduction efficiency and kinetics of Hph-1-ctCTLA-4 were analyzed by intracellular staining using fluorescein-labeled antibody to hemagglutinin. Left panel, after 1 h transduction, doses of 10 μ M (red), 5 μ M (green), 1 μ M (blue) showed intracellular presence of Hph-1-ctCTLA-4, whereas ctCTLA-4 was not detected inside cells even at a dose of 10 μ M (orange). Right panel, Hph-1-ctCTLA-4 transduced into Jurkat cells in time-dependent manner (15 min, blue; 30 min, green; 60 min, red; 120 min, yellow), whereas ctCTLA-4 was not detected even after 120 min of incubation (orange). **(c)** Hph-1-ctCTLA-4 inhibited expression of CD69 and CD25 on the activated Jurkat T cells. After preincubation of Hph-1-ctCTLA-4 with Jurkat cells for 60 min, cells were activated with CD3-specific and CD28-specific monoclonal antibodies. Expression levels of CD69 and CD25 were analyzed by flow cytometry. **(d-f)** Inhibition of IL-2 secretion by Hph-1-ctCTLA-4 in activated T cells. Human primary CD4⁺ or CD8⁺ T cells were purified by MACS. Hph-1-ctCTLA-4, control ctCTLA-4 and Hph-1-ctCTLA-4YF were transduced into Jurkat T cells and human primary CD4⁺ or CD8⁺ T cells followed by activation with plate-bound CD3-specific and CD28-specific monoclonal antibodies. IL-2 level in the supernatant from activated cells was analyzed by ELISA. As positive controls, cells were treated with 10 nM of CsA or FK506 for 60 min. Bars represent mean $\pm$ s.e.m. of three independent experiments. * P < 0.05, ** P < 0.01 compared with activated T-cell group.

Jurkat, human CD4⁺ or CD8⁺ T cells inhibited the induction of IL-2 secretion in a concentration-dependent manner at the picomolar level, and the level of inhibition by 10 pM Hph-1-ctCTLA-4 was better than or equivalent to that caused by 10 nM of cyclosporin A (CsA; **Fig. 2d-f**). Neither ctCTLA-4 without Hph-1-PTD nor Hph-1-ctCTLA-4YF, however, blocked secretion of IL-2. These results show that the delivery of ctCTLA-4 by Hph-1-PTD effectively blocks T-cell activation at 10 pM and that two tyrosines in ctCTLA-4 are essential for its inhibitory function^{18,19}.

To determine whether the Hph-1-ctCTLA-4-EGFP fusion protein can be efficiently delivered into the trachea and lung by intratracheal instillation in mice, we collected cells from bronchoalveolar lavage (BAL) fluid 30 min after intranasal administration of Hph-1-ctCTLA-4-EGFP. A marked number of BAL cells were positive for EGFP, and *in vivo* transduction efficiency of intranasally administered Hph-1-ctCTLA-4-EGFP was dose dependent (**Fig. 3a**). A high level of EGFP was detected in the majority of CD3⁺ T cells from mice intranasally administered Hph-1-ctCTLA-4-EGFP (**Fig. 3b**). EGFP fluorescence was detected only in trachea and lungs, whereas intranasally administered Hph-1-ctCTLA-4-EGFP was detected in neither nose nor in spleen, liver and intestine (**Fig. 3c,d**).

To investigate whether intranasally administered Hph-1-ctCTLA-4 is capable of suppressing the development of bronchial asthma *in vivo*, we generated a mouse model of ovalbumin (OVA)-induced asthma. Intranasal pretreatment of these mice with 25 μ g Hph-1-ctCTLA-4 strongly prevented the infiltration of inflammatory cells such as eosinophils, macrophages, neutrophils and lymphocytes into the BAL fluid (**Fig. 4a**). A single-dose pretreatment of OVA-challenged mice with 25 μ g Hph-1-ctCTLA-4 inhibited the production of eotaxin and TH2 cytokines, but it did not affect the secretion of interferon

(IFN)- γ and transforming growth factor (TGF)- β (**Fig. 4b**) and the level of serum IgE decreased significantly (**Fig. 4c**). Treatment with Hph-1-ctCTLA-4 prevented the infiltration of inflammatory cells into the peribronchial tissues, and very few goblet cells were detected around the bronchial airways (**Fig. 4d**). A significantly high level of inflammatory cell infiltration, however, and many periodic acid-Schiff (PAS)-positive and mucus-containing goblet cells around the bronchial airways were detected in the ctCTLA-4-only and Hph-1-ctCTLA-4YF mutant-treated groups. The semiquantitative value of the 'inflammatory index' from H&E-stained images (**Fig. 4e**) and the percentage of PAS-positive goblet cells (**Fig. 4f**) were significantly reduced in the OVA-challenged mice pretreated with Hph-1-ctCTLA-4. From these results, we conclude that the delivery of ctCTLA-4 using Hph-1-PTD can inhibit not only airway inflammation but also mucus metaplasia.

The level of OVA-induced hyper-responsiveness to methacholine was substantially attenuated by Hph-1-ctCTLA-4 (**Fig. 4g**). To confirm that the changes in airway responsiveness to methacholine measured by the enhanced-pause method were caused by changes in the airways of the trachea and lung, but not simply by nonspecific changes of airway resulting from OVA challenge and intranasal delivery of Hph-1-ctCTLA-4, we measured lung resistance (R_L) in ventilated mice (**Fig. 4h**). By this direct measurement of lung resistance, airways hyper-responsiveness was effectively normalized in mice treated with Hph-1-ctCTLA-4.

Here, we identified a novel PTD with the length of 11 amino acids from human transcription factor Hph-1. Hph-1-PTD has the capacity to efficiently and readily facilitate the transduction of a large protein *in vitro* and *in vivo*. Cell viability was not affected, even by a concentration 1,000-fold higher (10 mM) than the effective

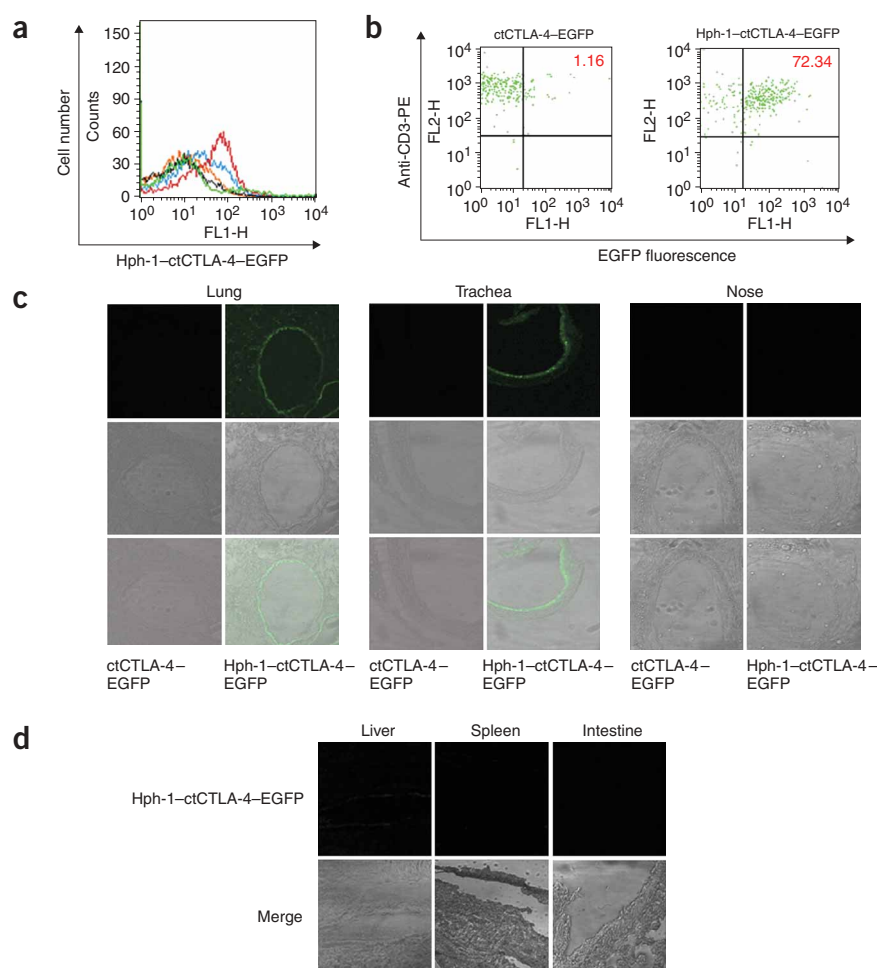


Figure 3 *In vivo* transduction of Hph-1-ctCTLA-4-EGFP through intranasal route. **(a)** Transduction of Hph-1-ctCTLA-4-EGFP into BAL cells after nasal instillation. After 5 (orange), 12.5 (blue), 25 (red) μ g Hph-1-ctCTLA-4-EGFP or ctCTLA-4-EGFP (green) was administered through the nasal route for 30 min, the levels of EGFP in the BAL cells were analyzed by FACS. **(b)** Hph-1-ctCTLA-4-EGFP effectively transduced into CD3⁺ T cells in the lung. The percent of CD3-EGFP double-positive cells was calculated. **(c,d)** Distribution of intranasally delivered Hph-1-ctCTLA-4-EGFP in lung, trachea, nose, liver, spleen and intestine was analyzed by confocal microscopy.

role of the CTLA-4 tyrosine motif in intracellular signaling for T-cell activation^{9–12}.

A recent study showed that expression of a B7 ligand–nonbinding CTLA-4 molecule inhibited the *in vitro* response of CTLA-4–knockout T cells and protected mice from the massive hyper-proliferation and tissue infiltration observed in CTLA-4-deficient mice²⁰. In another report, overexpression of the ligand-independent form of CTLA-4 (li-CTLA-4) inhibited T-cell activation in TKO (B7.1, B7.2, *Ctla4*^{–/–}) mice²¹. Therefore, the possible mechanism underlying the *in vivo* inhibitory effect of Hph-1-ctCTLA-4 on allergic inflammation is that the presence of ctCTLA-4 might block the T_H2 cell-polarized T-cell activation, leading to the prevention of infiltration of inflammatory cells. Intranasal administration of Hph-1-ctCTLA-4 enabled us to overcome the lack of tissue specificity of cationic PTDs, which limits their *in vivo* utility. Such reagents, and others that use the cytoplasmic domain of other surface receptors, could be promising therapeutics for the treatment of disease.

METHODS

Purification of Hph-1-PTD conjugated proteins.

We induced BL21 star (Novagen) transformed with each DNA construct for 5 h with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) and sonicated it in lysis buffer (6 M urea, 20 mM Tris-HCl, pH 8.0, and 500 mM NaCl). We clarified lysates by centrifugation (13,000 r.p.m. for 20 min at 4 °C) and loaded them on HisTrap chelating columns (Qia-gen). We washed bound proteins and eluted them with 50, 100, 250 mM or 3 M imidazole with >95% purity. We desalted the eluted proteins using PD-10 Sephadex G-25 (Amersham Pharmacia), supplemented them with 10% glycerol, separated them into aliquots and flash-froze them at –80 °C²².

Analysis of the *in vitro* and *in vivo* transduction efficiency of Hph-1-PTD.

We immediately fixed several cell lines and mouse tissues treated with Hph-1- β -gal and assayed them using an X-Gal staining kit (Roche)¹⁴. The image was captured by fluorescence microscopy (Nikon). To monitor the localization of Hph-1-EGFP, we incubated HeLa cells with 10 μ M Hph-1-EGFP for 30 min and detected fluorescence with confocal microscopy (Bio Rad). We analyzed the transduction efficiency of Hph-1-ctCTLA-4 by intracellular staining using fluorescein-labeled antibody to hemagglutinin (Cell Signaling).

Local administration of Hph-1-FITC. For skin, after we swept hairless mice with acetone, we applied a mixture of Hph-1-FITC and ointment to the backs of the mice for 2 h. The skin samples were analyzed by confocal microscopy. For eyes, we dropped Hph-1-FITC on the eyes of rats, and at 30 and 120 min after administration, we examined FITC fluorescence with confocal microscopy. For nasal airway, we intratracheally administered Hph-1-FITC into trachea and lung. After 30 min, we fixed the left main lung and trachea and analyzed them with confocal microscopy.

Analysis of T-cell activation. We purified primary CD4⁺ or CD8⁺ T cells from human peripheral blood mononuclear cells by MACS column using CD4 or CD8 microbeads (Miltenyi Biotec)²³. We treated 1×10^6 cells with

concentration of Hph-1-PTD; and behavioral abnormalities, cytotoxic effects and immunogenicity were not observed in mice injected intraperitoneally with 1.6 mg/kg or 100 μ g/mouse of Hph-1-ctCTLA-4, respectively (Supplementary Figs. 2 and 3 and Supplementary Table 1 online). Picomolar concentrations of Hph-1-ctCTLA-4 effectively inhibited secretion of IL-2 in Jurkat, primary human CD4⁺ and CD8⁺ T cells. The local delivery of Hph-1-ctCTLA-4 through the airway effectively suppressed both local inflammation in the airway and hyper-responsiveness.

With respect to *in vivo* stability, we observed that the therapeutic effects of Hph-1-ctCTLA-4 lasted at least 5–8 d in the OVA-induced experimental asthma model (Supplementary Figs. 4 and 5 online). Therapeutic efficacy was confirmed in another experimental airway inflammation model induced by toluene diisocyanate (TDI, Supplementary Fig. 6 online). These *in vitro* and *in vivo* inhibitory effects of Hph-1-ctCTLA-4 were not observed upon delivery of Hph-1-ctCTLA-4YF thus confirming that the inhibitory effects of Hph-1-ctCTLA-4 are specific to ctCTLA-4 and consistent with the known

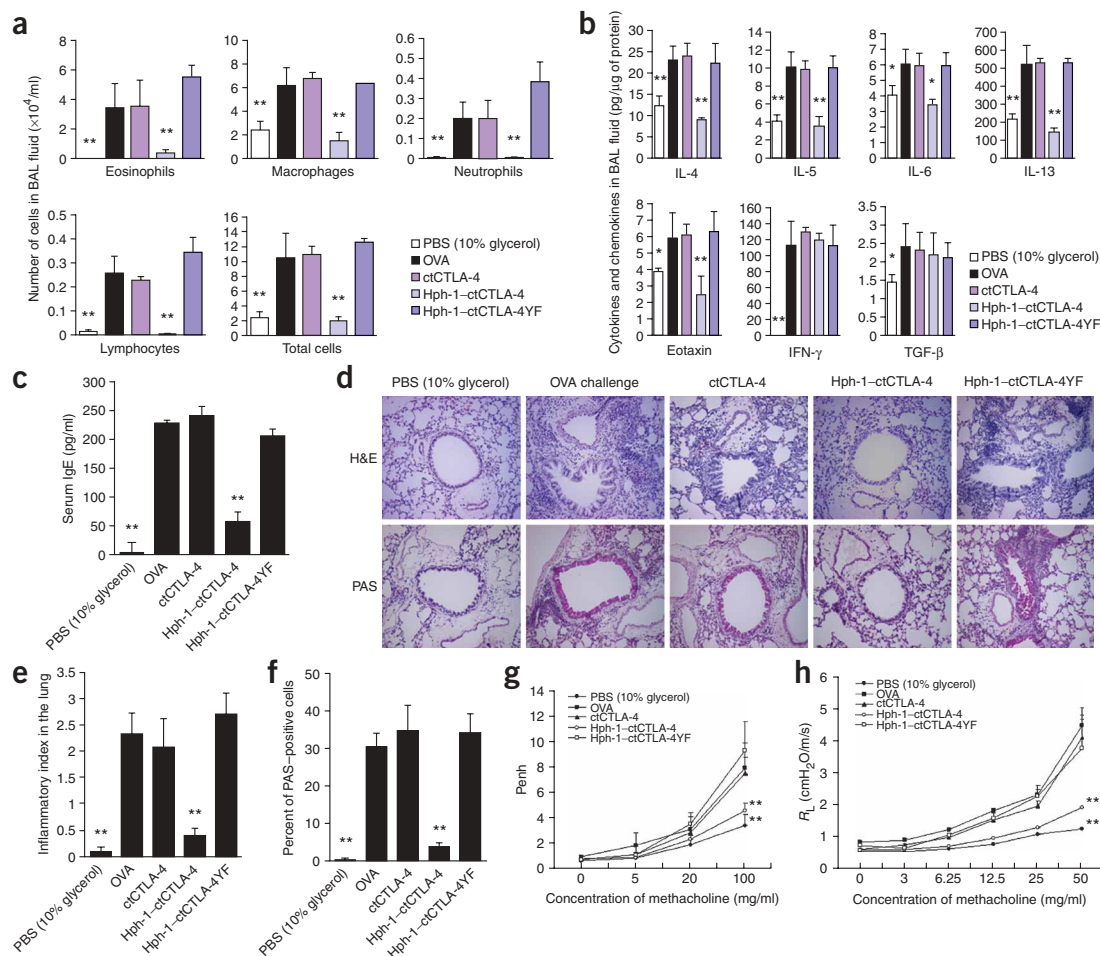


Figure 4 Therapeutic effect of nasal administration of Hph-1-ctCTLA-4 on airway inflammation in OVA-induced experimental mouse model of asthma. (a) Inhibition of the infiltration of inflammatory cells by Hph-1-ctCTLA-4. After nasal delivery of Hph-1-ctCTLA-4, ctCTLA-4 or Hph-1-ctCTLA-4YF through airway, the number of BAL cells was counted. (b) Suppression of T_H2 cytokines and chemokine secretion by Hph-1-ctCTLA-4 after OVA challenge. The levels of cytokines and chemokine secreted into BAL fluid were analyzed by ELISA. (c) Decrease of serum IgE level by Hph-1-ctCTLA-4. Level of OVA-specific IgE in mouse serum was analyzed by ELISA. (d) Histological examination of lung tissues. The lung sections were obtained from PBS- or OVA-challenged mice pretreated with Hph-1-ctCTLA-4, ctCTLA-4 or Hph-1-ctCTLA-4YF. Sections were stained with H&E for the analysis of tissue morphology and the level of inflammation. Sections were also stained with PAS to determine the presence of goblet cells. (e) Semiquantitative analysis of the severity of peribronchial inflammation. All of the randomly selected histological images were scored as the mean of inflammatory index. (f) PAS-positive cells in epithelium and total epithelial cells were counted, and the percentage of PAS-positive cells was calculated. (g) Airways hyper-responsiveness was measured as Penh (enhanced pause) in mice pretreated with Hph-1-ctCTLA-4, ctCTLA-4 or Hph-1-ctCTLA-4YF after challenge with increasing concentrations of methacholine. (h) Airways hyper-responsiveness was measured as average lung resistance (R_L) in anesthetized ventilated mice. Data are mean $\pm$ s.e.m. of 6–15 mice. * $P < 0.05$, ** $P < 0.01$ compared with the OVA-only-challenged group.

Hph-1-ctCTLA-4 for 1 h, then activated them with 0.25 μ g soluble CD28-specific monoclonal antibody (Pharmingen) and 0.05 μ g plate-bound CD3-specific monoclonal antibody (Pharmingen) for 24–48 h. We stained cells with PE-labeled antibody to CD69 or FITC-labeled antibody to CD25 (Pharmingen) and analyzed them by FACSCalibur (Becton Dickinson). We performed an ELISA to analyze IL-2 in supernatant (R&D Systems).

OVA-induced allergic airway inflammation model. We maintained 6-week-old male BALB/c (Charles River Technology) mice under pathogen-free conditions. Mouse care and experimental procedures were performed under approval from the Animal Care Committee of Yonsei University and Soonchunhyang University. We sensitized mice on days 1 and 14 by intraperitoneal injection of 100 μ g OVA (Sigma) emulsified in 20 mg aluminum hydroxide (Sigma). On days 15, 16 and 17 after the initial sensitization, we challenged mice with 1.5 mg OVA^{24,25}. Mice received 25 or 5 μ g Hph-1-ctCTLA-4 30 min before intranasal OVA challenge.

TDI-induced airway inflammation model. We intranasally administered 20 μ l 3% TDI dissolved in ethyl acetate plus olive oil (1:4 dilution) to mice once daily for 5 consecutive days. Four days after sensitization, we challenged mice with 1% TDI by ultrasonic nebulization for 1 h on 3 consecutive days, according to a previously described method²⁶ with slight modification.

Analysis of inflammation in airway. We counted BAL cells using a cyto-centrifugation and Diff-Quick staining (Scientific Products). We subjected trachea and left main lung to H&E staining and to PAS (periodic acid-Schiff) staining. The severity of peribronchial inflammation was graded semiquantitatively as previously described: 0, normal; 1, fewer cells; 2, a ring of inflammatory cells one cell deep; 3, a ring of inflammatory cells more than four cells deep^{27,28}. We counted PAS-positive epithelial cells and total epithelial cells using software (Nikon DXM 1200, Nikon Inc. and Image Pro Plus 4.01 software, Media Cybernetics). The levels of eotaxin, IL-4, IL-5 (BD Biosciences), IL-6 and IL-13 (R&D Systems) in the BAL fluid and IgE (BD Biosciences) in the serum were measured by ELISA.

Airway hyper-responsiveness. Method 1: Measurement of airway responsiveness in conscious, spontaneously breathing mice. We measured airways hyper-responsiveness in unrestrained mice by noninvasive whole-body plethysmography (All Medicous, Inc.). We used the change in chamber pressure as a measure of the difference between thoracic volume expansion and contraction, and between air volume removed and added to the chamber during respiration²⁹. Method 2: Measurement of airway responsiveness in anesthetized ventilated mice. Airways hyper-responsiveness was assessed as a change in airway resistance (cmH₂O/ml/s) after challenge with aerosolized methacholine (MCh). Anesthetized (pentobarbital sodium, 70–90 mg/kg, intraperitoneal), tracheostomized (stainless steel cannula, 18 G) mice were mechanically ventilated, and lung function was assessed using methods described previously³⁰. Lung resistance (R_L) and compliance were measured using FlexiVent system (Scireq).

Toxicity and immunogenicity of Hph-1-ctCTLA-4 in mice. We intravenously injected 1.6 mg/kg of Hph-1-ctCTLA-4 into six mice every day for 14 d. We monitored weight change, lethality and general abnormality, and examined levels of aspartate aminotransferase, alanine aminotransferase, blood urea nitrogen and creatinine in the blood to assess liver and kidney toxicity. To investigate the immunogenicity of Hph-1-ctCTLA-4, we injected mice intraperitoneally with 100 µg Hph-1-ctCTLA-4 or OVA two times a week for 2 weeks. The total IgG and antigen-specific IgG in the serum were examined by ELISA.

Statistical analysis. Data are presented as mean ± s.d. Statistical analysis of group differences was examined using the nonparametric Mann-Whitney U-test. *P < 0.05 and **P < 0.01 were considered to be significant.

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have competing financial interests (see the *Nature Medicine* website for details).

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