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*J Immunol* 2007; 179:2556-2564; doi: 10.4049/jimmunol.179.4.2556
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Inhibition of Experimental Allergic Airways Disease by Local Application of a Cell-Penetrating Dominant-Negative STAT-6 Peptide

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Allergic airways disease is initiated and perpetuated by an aberrant Th2 inflammatory response regulated in part by the cytokines IL-4 and IL-13, each of which induces activation of the STAT-6 transcription factor. Data from murine models indicate that the clinical manifestations of acute asthma are STAT-6 dependent, and thus, STAT-6 is a target for drug development in allergic airways disease. We designed a novel chimeric peptide (STAT-6 inhibitory peptide (STAT-6-IP)) comprised of a sequence predicted to bind to and inhibit STAT-6, fused to a protein transduction domain, to facilitate cellular uptake of the STAT-6-binding peptide. Our data demonstrate that the STAT-6-IP inhibited OVA-induced production of Th2 cytokines IL-4 and IL-13 in vitro. In contrast, the STAT-6-IP did not affect production of IFN-γ, demonstrating specificity for Th2 cytokine inhibition. Following intranasal administration, the STAT-6-IP was localized to epithelial cells in the airways. Finally, in in vivo murine models of allergic rhinitis and asthma, intranasal delivery of the STAT-6-IP inhibited OVA-induced lung inflammation and mucus production as well as accumulation of eosinophils and IL-13 in bronchoalveolar lavage fluid and OVA-dependent airway hyperresponsiveness. Together these data show that local application of cell-penetrating peptide inhibitors of STAT-6 has significant potential for the treatment of allergic rhinitis and asthma. The Journal of Immunology, 2007, 179: 2556–2564.

Asthma and rhinitis are diseases affecting between 20 and 30% of the population with significant morbidity (1, 2). In predisposed individuals, exposure of the airways to otherwise innocuous aeroallergens results in activation of Ag-specific CD4+ T lymphocytes of the Th2 phenotype and secretion of specific cytokines, including IL-4 and IL-13 (3, 4). Associated with Th2-driven inflammation, the airways of asthmatics are characterized by increased mucus secretion, epithelial shedding, microvascular leakage, remodeling, and hyperresponsiveness (5). Binding of IL-4 or IL-13 to their cognate receptors, each of which includes the IL-4Rα subunit, induces activation of the cytokine receptor-associated tyrosine (Tyr)3 kinases Jak 1, Jak 3, and Tyk 2 (6), which in turn phosphorylate specific Tyr residues on the IL-4Rα subunit (7, 8). Cytoplasmic STAT-6 is recruited to the phosphorylated receptor via the STAT-6 Src homology 2 (SH2) domain, whereupon Tyr641 (Y641) of STAT-6 is phosphorylated (phosphotyrosine (*Y) 641) (7, 8). Two phosphorylated STAT-6 molecules subsequently homodimerize (via paired *Y641-SH2 domain interactions) and translocate to the nucleus, where they regulate IL-4/IL-13-dependent gene expression (9).

Consistent with a role for IL-4 and IL-13 in asthma pathogenesis, data from murine models of acute experimental asthma indicate that STAT-6 knockout mice do not develop the characteristic airway hyperresponsiveness (AHR) and lung pathology associated with asthma (10, 11). Although the inability of T cells in STAT-6 knockout mice to differentiate into Th2 effector cells is almost certainly critical, the lack of responsiveness in these animals may also be due at least in part to the absence of STAT-6 activity in airway structural cells. Data from Luster and colleagues (12) demonstrate that T cell-specific STAT-6 expression is not sufficient for pathogenesis in experimental asthma and that lung specific STAT-6 expression regulates airway inflammation, mucus production, and AHR. Consistent with these data, mucus production and AHR are intact in IL-13 transgenic mice in which STAT-6 expression is limited to airway epithelial cells, providing evidence that epithelial cell-specific STAT-6 activity is sufficient to drive two of the main pathologic features of experimental murine asthma (13).

STAT-6 is thus an attractive therapeutic target for management of allergic airways disease, not only because it regulates differentiation of Th2 cells, but also because it directly regulates IL-13-dependent responses that contribute to asthma pathogenesis. However, targeting STAT-6 is complicated due to its intracellular location. To circumvent this difficulty, we have exploited protein transduction technology to introduce into cells a specific peptide inhibitor of STAT-6 (STAT-6-IP). We hypothesized that a peptide containing amino acid residues surrounding *Y641 of STAT-6, when introduced into STAT-6-containing cells, would act in a dominant-negative fashion by binding to and inhibiting phosphorylated STAT-6. To overcome the challenge of intracellular peptide delivery, the STAT-6-binding peptide was coupled to protein...
transduction domain 4 (PTD4), a derivative of the HIV-TAT transcription factor protein transduction domain (14). We investigated the ability of this 19 aa cell-penetrating STAT-6-binding peptide to effectively inhibit STAT-6 activity both in vitro and in vivo.

There are several advantages to using cell-penetrating peptides to inhibit STAT-6 activity. Because the STAT-6-IP has a limited "t1/2," inhibition will most likely be transient and, thus, different from transgenic and knockout models, use of this peptide can provide information regarding the mechanism by which transient inhibition of STAT-6 regulates allergic airways disease. Moreover, the STAT-6-IP can be used to investigate the role of STAT-6 in experimental asthma in species for which knockout/transgenic technology is not a viable option. Finally, data obtained in animal models will provide evidence that the STAT-6-IP may offer an important novel therapeutic approach for the treatment of allergic airways disease in humans.

We demonstrate that the STAT-6-IP inhibited Th2 cytokine and chemokine production in cultured splenocytes and epithelial cells, respectively. When delivered selectively to the upper airways, the STAT-6-IP inhibited Ag-induced inflammatory responses and AHR. Moreover, when delivered directly to the lungs, the STAT-6-IP inhibited Ag-induced inflammatory responses, including chemokine expression, mucus production, and eosinophil influx into the lungs.

Materials and Methods

Peptide synthesis and uptake by splenocytes

STAT-6 peptides were synthesized by the University of Calgary Integrated Peptide Services or Biopeptide. The FITC-conjugated PTD4 peptide was synthesized by the Sheldon Biotechnology Center. Peptides were amidated at the carboxyl terminus and purified by reversed phase HPLC and analyzed by mass spectroscopy. The model of the STAT-6-binding portion of the STAT-6-IP presented in Fig. 1a was generated using HyperChem 7.5 from Hypercube.

To monitor peptide uptake, splenocytes in DMEM/10% FBS were incubated continuously with FITC-conjugated PTD4 peptide (100 \(\mu\)M) for 30 min to 24 h. At each time point, surface-bound peptide was removed by washing cells twice with PBS and then incubating cells with trypsin for 5 min. Ten thousand cells were subsequently analyzed by flow cytometry. To monitor the "t1/2" of intracellular peptide (in the absence of uptake), splenocytes in DMEM/10% FBS were incubated with FITC-conjugated PTD4 peptide (100 \(\mu\)M) for 4 h, after which they were washed twice with PBS and trypsinized. Splenocytes were then incubated with soybean trypsin inhibitor (0.5 mg/ml in 10 mM sodium tetraborate) for 5 min. The cells were washed again once in PBS and subsequently placed back into complete medium. At different time points (as indicated in the figure legends), cells were harvested and lysed in tricine sample buffer (100 mM Tris-HCl (pH 6.8), 24% glycerol, 8% SDS, and 200 mM DTT). Lysates were clarified, and equal concentrations (20 \(\mu\)g/lane) were resolved by SDS-PAGE on 18% Tris-tricine gels, after which proteins were transferred to polyvinylidene difluoride membrane. For Western immunoblotting, membranes were blocked for 1 h in TBST (10 mM Tris-Cl (pH 7.4), 2.5 mM EDTA, 150 mM NaCl, and 0.1% Tween 20) containing 0.25% gelatin (bloom 275) at room temperature. Blots were incubated with anti-phospho-STAT-6 (Tyr641) Ab and ECL. e. Mean values of three independent experiments are reported \(\pm\) SEM.

FIGURE 1. Model of STAT-6-IP and uptake of PTD4-containing peptides by primary murine splenocytes. a. Model of STAT-6-IP with the phosphotyrosine residue predicted to bind to phosphorylated STAT-6 oriented to the left. b. Fluorescence of splenocytes incubated for different times with the FITC-PTD4 peptide. c. Stability of FITC-PTD4 peptide in splenocytes assessed by monitoring fluorescence (by flow cytometry) of splenocytes incubated with FITC-PTD4 peptide, trypsinized, and placed back into complete medium for times indicated. d. Stability of STAT-6-IP in splenocytes. Cells were treated as in c, but at different time points cells were lysed and equal quantities of protein were resolved on Tris-tricine gels. STAT-6-IP was detected using anti-phospho-STAT-6 Ab and ECL. e. Mean values of three independent experiments are reported \(\pm\) SEM.
sacrificed and spleens were harvested. A single-cell suspension of splenocytes (5 × 10^6 cells/ml) was cultured in RPMI 1640 medium containing 10% heat-inactivated FBS supplemented with 50 μM 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 2 mM l-glutamine. Splenocytes were cultured for 4 days in the presence or absence of 100 μg/ml OVA, either alone or with 100 μM STAT-6-IP or STAT-6-negative control peptide (STAT-6-CP), which were added daily. No evidence of cellular toxicity has been found in either murine splenocytes or immortalized epithelial cells (data not shown). After 4 days, culture supernatants were harvested to quantify cytokine production by ELISA. Murine IL-4 and IFN-γ ELISA kits were purchased from BD Pharmingen, and murine IL-13 ELISA kit was purchased from BioSource International. To monitor expression of cytokines by intracellular cytokine staining, the splenocytes were cultured for 5 h with murine anti-CD3 (0.5 μg/ml; clone 2C11) and monensin (GolgiStop; BD Pharmingen), according to the manufacturer’s instructions. Cells were then washed, permeabilized with saponin (PermeaWash; BD Pharmingen), and fixed in formaldehyde and PBS (Cytofix/Cytoperm; BD Pharmingen) for 30 min. Splenocytes were stained with FITC-conjugated rat anti-mouse CD4, PE-conjugated rat anti-mouse IL-4, and allophycocyanin-conjugated rat anti-mouse IFN-γ (all from BD Pharmingen). Cytokine expression was monitored by flow cytometry and analyzed using CellQuest software (BD Biosciences). Experiments were replicated independently three times, and means are reported.

We also assessed the ability of the STAT-6-IP to inhibit production of eotaxin-3/CCL26 from human BEAS-2B epithelial cells. Cells were plated in 24-well cell culture plates in DMEM:F12 medium containing 10% heat-inactivated FBS supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin sulfate. When cells were ~90% confluent, they were washed twice with PBS and placed into starvation medium comprised of DMEM:F12 containing 0.1% BSA. After 24 h of starvation, IL-4 (1 ng/ml) was added to cells, a subset of which had been previously loaded for 2 h with 100 μM STAT-6-IP or STAT-6-CP. Subsequently, peptides were added daily, and eotaxin-3/CCL26 production at time 0 (before adding IL-4) and 24, 48, 72, and 96 h was quantified from cell culture supernatants using an ELISA kit from R&D Systems.

**Animals**

BALB/c mice (6–8 wk old) were obtained from Harlan Sprague-Dawley and were housed in a conventional animal facility at the Meakins-Christie Laboratories. For each experimental condition, a minimum of five to eight animals was used. In some experiments, mice were exposed intranasally to OVA and peptides while awake to deliver OVA and peptides selectively to the upper airways (16–18). These mice were sensitized 5 days per week for each of 2 wk by intranasal instillation of 100 μg of OVA in PBS. Following a 2-wk rest period, mice were challenged with OVA and peptides while awake to deliver OVA and peptides selectively to the upper airways (16–18). These mice were sensitized 5 days per week for each of 2 wk by intranasal instillation of 100 μg of OVA in PBS. Following a 2-wk rest period, mice were challenged with OVA and peptides while awake to deliver OVA and peptides selectively to the upper airways (16–18). These mice were sensitized 5 days per week for each of 2 wk by intranasal instillation of 100 μg of OVA. Mice were similarly sensitized mice were challenged with OVA and peptides under anesthesia, to promote delivery of OVA and peptides to the lower airways (17, 19). Mice were sensitized 5 days per week for each of 2 wk by intranasal instillation of 100 μg of OVA.

After a 2-wk rest, mice were briefly anesthetized with isofluorane before being challenged with 10 μg of OVA daily for each of 5 days. Peptides were delivered to mice intranasally before each OVA challenge. AHR was assessed and bronchoalveolar lavage (BAL) fluid was collected (see below) 24 h after the final challenge. In other experiments, OVA-sensitized mice were challenged with OVA and peptides under anesthesia, to promote delivery of OVA and peptides to the lower airways (17, 19). Mice were sensitized 5 days per week for each of 2 wk by intranasal instillation of 100 μg of OVA. Mice were similarly sensitized mice were challenged with OVA and peptides under anesthesia, to promote delivery of OVA and peptides to the lower airways (17, 19). Mice were sensitized 5 days per week for each of 2 wk by intranasal instillation of 100 μg of OVA. Mice were similarly sensitized mice were challenged with OVA and peptides under anesthesia, to promote delivery of OVA and peptides to the lower airways (17, 19). Mice were sensitized 5 days per week for each of 2 wk by intranasal instillation of 100 μg of OVA.
To assess AHR, 24 h after the final OVA challenge, mice were deeply anesthetized with xylazine and sodium pentobarbital and paralyzed with pancuronium bromide. Heart rate was monitored by electrocardiogram. The trachea was exposed, and a cannula was inserted, secured with silk ties, and then attached to a computer-controlled small-animal ventilator (FlexiVent; SCIREQ). Mice were ventilated quasisinusoidally with a tidal volume of 8 ml/kg and respiratory rate of 150 breaths/min. Baseline respiratory system resistance was measured. Metacholine was injected through the tail vein, and the maximal resistance was recorded, as described previously (16, 18). To perform BAL, the trachea was isolated and a 22-gauge stainless steel catheter was inserted into the proximal trachea and secured with 4-0 silk suture. The lungs were lavaged using ice-cold 0.9% NaCl. The BAL fluid was centrifuged to remove intact cells, and the supernatant was stored at −20°C for IL-13 analysis by ELISA (see below). RBC in the BAL fluid cell pellet were lysed. Cells were spun onto glass slides and stained using Diff-quick (Fisher Scientific), and differential cell counts were obtained manually under light microscopy. Three to six fields, each comprised of 100 cells, were counted per slide, and means were obtained. To quantify absolute cell numbers recovered in the BAL fluid, live cells that excluded trypan blue were counted before cytopsins. All animal studies were approved by the McGill University Animal Care Committee and performed following the guidelines of the Canadian Council on Animal Care.

**Immunofluorescence and histological analyses of lung tissue and BAL fluid cytopsins**

Histology was performed, as described previously (16). Briefly, lungs were slowly inflated with 1 ml of formalin, isolated, and then placed entirely in formalin. The specimens were embedded in paraffin, and 0.5-μm sections were cut. To assess inflammation, slides were stained with Giemsa, and to assess mucus, slides were stained with periodic acid Schiff (PAS).

To detect in vivo uptake of STAT-6-IP, mice were briefly anesthetized with halothane and then exposed intranasally with 150 μg of FITC-conjugated STAT-6-IP in a volume of 40 μl. Four hours later, mice were sacrificed with an overdose of sodium pentobarbital, and lungs were lavaged with 2 ml of PBS, harvested, and fixed in 2% paraformaldehyde. Immunofluorescence of lung sections was detected using an Olympus BX 51 Fluorescence Microscope System (Olympus). Images were then analyzed using Image-Pro Plus 4.5 software (Media Cybernetics).

**Real-time RT-PCR**

To assess RNA levels encoding eotaxin-1/CCL11, muc2, and muc5ac in the lung, total cellular RNA was extracted from the lung using an RNaseasy Mini Kit, according to the manufacturer’s protocol (Qiagen), and reverse transcribed using SuperScript II reverse transcriptase (Invitrogen Life Technologies). Real-time quantitative PCR was performed with a LightCycler System (Roche Applied Sciences) using Quantitec SYBR Green (Qiagen). Each target was quantified using four 100-fold serial dilutions of standards prepared from PCR amplicons that had been gel purified and quantified spectrophotometrically. Values were then normalized to GAPDH that had been reverse transcribed, PCR amplified, and quantified in the same manner. The following specific primers were used:

- **muc2**, 5′-GCT GAC GAG TGG TGT GTG AAT G-3′ and 3′-GAT GAG GCA GAC AGA C-3′; 
- **muc5ac**, 5′-CAG CCG AGA GGA GGG TTT GAT CT-3′ and 3′-AGT CTC TCT CCG CTC TCT TCA AT-3′; 
- **eotaxin-1/CCL11**, 5′-GGG CAG TAA CTT CCA TCT GTC TCC-3′ and 3′-CAC TTC TTC TGG GTC GC-3′; and 
- **GAPDH**, 5′-GCC ATG GAC TGT GAT CAT GA-3′ and 3′-TTC ACC ACC ATG GAG AAG GC-3′.

**Statistical analysis**

The results are expressed as mean ± SEM. Statistical significance was measured by one-way ANOVA, followed by Tukey’s posthoc tests for individual group comparisons using SPSS software. Individual comparisons for each data set are reported in the figure legends. A value of *p* < 0.05 was considered significant.

**Results**

The **STAT-6-IP efficiently enters cells in vitro**

To inhibit intracellular STAT-6 activity, we designed a cell-penetrating STAT-6-binding peptide (STAT-6-IP) in which the HIV-TAT-derived PTD4 protein transduction domain (YARAARQARAGRFVSTT) was fused to 7 aa surrounding *Y641* of murine STAT-6 (YARAARQARAGRFVSTTYVSTT) (Fig. 1a). A negative control peptide, STAT-6-CP, in which *Y641* was replaced by a phenylalanine residue (F), was also synthesized (YARAARQARAGRFVSTT). The ability of the PTD4 protein transduction domain to enter cells was confirmed by incubating murine splenocytes with FITC-conjugated PTD4 peptide (FITC-PTD4). Splenocytes were incubated with FITC-PTD4 for various times (30 min to 24 h), subsequently washed and incubated with trypsin to remove surface-bound peptide, and analyzed by flow cytometry. Half-maximal peptide uptake occurred at 1 h and plateaued after 4–6 h (Fig. 1b). To determine the $t_{1/2}$ of the intracellular PTD4
peptide in the absence of further uptake, splenocytes were incubated with the FITC-PTD4 peptide for 4 h, washed, trypsinized, and placed back into complete medium, after which intracellular localization of the peptide was monitored over time by flow cytometry. Under these conditions, intracellular FITC-PTD4 had a $t_{1/2}$ of $\sim 4$ h, although the peptide was detectable in the splenocytes for up to 24 h (Fig. 1c). To monitor stability of the STAT-6-IP in murine splenocytes, cells were incubated with the STAT-6-IP for 4 h, after which they were washed and trypsinized. The presence of intracellular STAT-6-IP was monitored by Western blotting of cell lysates harvested at different time points. The STAT-6-IP had a $t_{1/2}$ of $\sim 8$ h and, similar to the FITC-PTD4 peptide, remained in the cells for up to 24 h. Together these data demonstrate that the PTD4 protein transduction domain entered cells and facilitated intracellular uptake of the STAT-6-IP.

**FIGURE 4.** The STAT-6-IP significantly reduces lung inflammation in experimental rhinitis and mild asthma. BALB/c mice were sensitized, challenged, and treated with STAT-6-IP or STAT-6-CP as in Fig. 3. Lung tissue was harvested, and inflammation was assessed in Giemsa-stained lung sections at $\times 400$ magnification. a, Mice exposed to saline during both the sensitization and challenge phases; b, mice exposed to OVA during both the sensitization and challenge phases; c, mice were sensitized with OVA, and then before each OVA challenge animals received 150 $\mu$g of STAT-6-IP (IP); d, mice were sensitized with OVA, and then before each OVA challenge animals received 150 $\mu$g of STAT-6-CP (CP). Arrows indicate eosinophils.

**FIGURE 5.** The STAT-6-IP is detected in structural cells of the airways following intranasal administration. FITC STAT-6-IP or control vehicle was administered intranasally to BALB/c mice. Peptide- and control-treated mice were then sacrificed 4 h later, at which point lungs were inflated with OCT compound in PBS and frozen. Bright field and fluorescent images from lung tissue sections were then captured with an Olympus BX 51 fluorescence microscope system and analyzed using Image-Pro Plus 4.5 software. Equivalent exposure times were used for control (a–c)- and FITC STAT-6-IP (d–f)-treated animals.
The STAT-6-IP inhibits OVA-induced Th2 cytokines in vitro

We then postulated that treatment of splenocytes from OVA-sensitized mice with the STAT-6-IP would inhibit STAT-6 and thus decrease OVA-induced production of Th2 cytokines (15). To test this hypothesis, splenocytes from OVA-sensitized mice were cultured for 4 days with no Ag, with OVA alone, or with OVA plus the STAT-6-IP or STAT-6-CP. The STAT-6-IP and STAT-6-CP were added to splenocyte cultures daily. Treatment with the STAT-6-IP reduced the supernatant levels of both IL-4 and IL-13 (Fig. 2, a and b), whereas the STAT-6-CP did not significantly inhibit OVA-induced production of IL-4 or IL-13. Neither peptide inhibited OVA-induced IFN-γ production (Fig. 2c). These data suggest that the STAT-6-IP specifically inhibited STAT-6-dependent activity. We also quantified the number of CD4+ T cells producing IL-4 by intracellular cytokine staining. When splenocytes from OVA-sensitized mice were cultured with OVA, there was a significant increase in the number of CD4+ lymphocytes expressing IL-4 (Fig. 2, d and e). The increase in IL-4 expression was abrogated in splenocytes cultured with OVA in the presence of the STAT-6-IP, whereas the STAT-6-CP had no effect. Altogether, these data indicate that the STAT-6-IP efficiently entered cells and selectively inhibited Th2 cytokine production, leaving Th1 cytokine production intact.

The STAT-6-IP inhibits OVA-induced inflammation, mucus production, and AHR in vivo

We assessed the inhibitory activity of the STAT-6-IP in a murine model of allergic airways disease we developed to mimic events that occur in the development of human atopy (16). In this model, nonanesthetized mice are sensitized and challenged with OVA intranasally, the primary route of allergen exposure in humans. In nonanesthetized mice, OVA deposition occurs almost exclusively in the upper airways (17, 19). Mice sensitized in this manner produce OVA-specific IgE, and upon intranasal OVA challenge to the upper airways, develop moderate inflammation and eosinophilia in both the upper and lower airways as well as robust IL-13-dependent AHR (16, 18). This model is consistent with epidemiological studies showing that the large majority of patients with asthma have intermittent or mild persistent disease (20) and that up to 30% of patients with physician-diagnosed allergic rhinitis have asthma symptoms (21). Treatment of these OVA-sensitized mice before OVA challenge with the STAT-6-IP, but not the STAT-6-CP, inhibited, in a dose-dependent manner, allergen-induced accumulation of granulocytes, quantified in BAL fluid (Fig. 3a). The STAT-6-IP also decreased the OVA-induced increase of IL-13 in the BAL fluid (Fig. 3b), the highest dose of STAT-6-IP inhibiting IL-13 to baseline levels. AHR was also inhibited by the STAT-6-IP (Fig. 3c). Histology of lungs revealed that the OVA-induced lung inflammation, including airway eosinophilia, was reduced in animals treated with the STAT-6-IP, whereas lung inflammation in mice that received the STAT-6-CP did not differ from that in OVA-sensitized and -challenged animals (Fig. 4).

We then investigated the ability of the STAT-6-IP to inhibit Th2-dependent allergic responses to inhaled allergen in the lung in a model of moderate to severe acute asthma. We first assessed the in vivo uptake of FITC-tagged version of the STAT-6-IP when delivered to the lungs. Our data demonstrate that the FITC-tagged STAT-6-IP was localized to epithelial cells of the airways (Fig. 5). Subsequently, the ability of the STAT-6-IP to inhibit Th2-dependent allergic responses to inhaled allergen in experimental asthma was assessed. The STAT-6-IP, but not the STAT-6-CP, inhibited...
OVA-induced accumulation of inflammatory cells, including eosinophils, in the lung (Fig. 6). Moreover, the STAT-6-IP, but not the STAT-6-CP, also inhibited OVA-induced expression of muc5ac (Fig. 7a), muc2 (Fig. 7b), and eotaxin-1/CCL11 (Fig. 7c) in the lungs of allergic mice. Consistent with its ability to inhibit expression of genes encoding mucus glycoproteins, the STAT-6-IP also inhibited OVA-induced increase in goblet cell metaplasia and mucus production in both the upper airways and lungs of mice exposed to inhaled OVA (Fig. 8). Altogether, these data demonstrate that the STAT-6-IP was localized to airway epithelial cells and effectively inhibited OVA-induced lung inflammation, mucus production, and AHR in experimental allergic airways disease.

The STAT-6-IP inhibits IL-4-induced production of eotaxin-3/CCL26 from cultured BEAS-2B epithelial cells

The localization of the STAT-6-IP to airway epithelial cells as well as its ability to inhibit OVA-induced expression of muc2, muc5ac, and eotaxin-1/CCL11 in the lungs of allergic mice suggested that the STAT-6-IP may inhibit asthma pathogenesis, at least in part, by inhibiting STAT-6 activity in epithelial cells. Thus, we assessed the ability of the STAT-6-IP to inhibit IL-4-induced production of eotaxin-3/CCL26 from cultured human BEAS-2B bronchial epithelial cells (22, 23). We used modified versions of the STAT-6-IP and STAT-6-CP in which the STAT-6 sequence of the peptides was derived from the human STAT-6 (hSTAT-6) protein (hSTAT-6-IP), YARAAARQARAGRG*YVPAT, and hSTAT-6-CP, YARAAARQARAGRGFVPAT. Our data demonstrate that in cells treated with the hSTAT-6-IP, but not the hSTAT-6-CP, IL-4-induced production of eotaxin-3/CCL26 was inhibited (Fig. 9). The ability of the STAT-6-IP to inhibit IL-4-induced eotaxin-3/CCL26 production was time dependent; inhibition was first seen at 72 h and increased to become statistically significant at 96 h.

Discussion

Abundant data support a role for Th2 cells and their cytokines in the pathogenesis of allergic rhinitis and asthma, both in humans and in animal models of allergic airway disease. Thus, Th2 cytokines, their receptors, and the transcription factors that mediate Th2 cytokine-specific cellular responses are targets for the treatment of allergic rhinitis and asthma. One therapeutic approach that has shown promise is to inhibit expression of the proteins that regulate asthma pathogenesis. Antisense oligonucleotides that inhibit expression of IL-4, the common α-chain shared by IL-5, IL-3, and GM-CSF receptors, or the Th2-specific GATA-3 transcription factor effectively inhibit airway inflammatory responses as well as AHR in experimental asthma (24–26). In addition to antisense technology, soluble cytokine receptor subunits have been used to sequester IL-4 or IL-13 to inhibit allergic asthma (27–30). As well, dominant-negative mutants of IL-4 and IL-13 effectively inhibit activation of IL-4R/IL-13R by the wild-type cytokines, and thus may also have therapeutic potential (31–33).

FIGURE 8. The STAT-6-IP reduces mucus production in allergic mice. BALB/c mice were sensitized with NS or OVA. Before each OVA challenge, mice were briefly anesthetized before receiving either NS (a, b, e, f), or STAT-6-IP (IP) (c and g), or STAT-6-CP (CP) (d and h). Animals were then challenged with NS (a and e) or OVA (b–d and f–h). Nares and lung tissue were harvested and imbedded in paraffin, and mucus-positive cells were identified by staining sections with PAS. Magnification is ×200.

FIGURE 9. The STAT-6-IP inhibits IL-4-induced eotaxin-3/CCL26 production from epithelial cells. BEAS-2B bronchial epithelial cells were loaded with 100 μM hSTAT-6-IP or hSTAT-6-CP for 2 h and then stimulated with IL-4 (1 ng/ml). Subsequently, peptides were added daily, and eotaxin-3/CCL26 production at time 0 (before adding peptides or IL-4) and 24, 48, 72, and 96 h was quantified from cell culture supernatants by ELISA. Mean values of eotaxin-3/CCL26 production from three independent experiments ± SEM are reported. * p < 0.05, compared with cells cultured with IL-4 or IL-4 + CP.
Inhibiting activity of the STAT-6 and GATA-3 transcription factors, which regulate Th2 cytokine-dependent gene expression, may also ameliorate allergic airways disease. However, because STAT-6 and GATA-3 are intracellular proteins, inhibition using exogenously delivered compounds is more complex and requires that inhibitory molecules have the ability to enter target cells. We have taken advantage of protein transduction technology to generate a cell-penetrating inhibitory peptide targeting STAT-6. The STAT-6-IP we designed is comprised of a sequence predicted to bind to and inhibit STAT-6, fused to a protein transduction domain, to facilitate cellular uptake of the STAT-6-binding peptide.

Protein transduction domains were first identified in HIV-TAT and Drosophila Antennapedia, two transcription factors with the ability to enter cells via a receptor-independent mechanism (34, 35). Several peptides, both naturally occurring and synthetic, are now known to possess this activity (34). Each of these domains is highly basic and appears to interact with the plasma membrane and/or cell surface glycosaminoglycans before entering cells via endocytosis (36–38). Protein transduction domains also have the exceptional ability to transport into cells various types of cargo, including nucleic acids, peptides, and even full-length proteins. Following transport into target cells, transducing peptides and their conjugated cargo are subsequently released, allowing the cargo to regulate cellular responses. Cell-penetrating proteins or peptides have been used successfully in a number of animal models of human disease (39–44).

We were interested in exploiting protein transduction technology to inhibit the SH2 domain containing STAT-6 transcription factor as a possible therapeutic strategy for allergic rhinitis and asthma. The STAT-6-IP we designed contains the sequence surrounding *Y641 from STAT-6 itself, which mediates homodimerization of activated STAT-6, and is thus predicted to preferentially inhibit STAT-6 that has already been Tyr phosphorylated in IL-4/IL-13-stimulated cells. Another cell-penetrating STAT-6-binding peptide has also been characterized (45). This peptide is comprised of the protein transduction domain from Drosophila Antennapedia coupled to the sequence surrounding *Y606 of the human IL-4Rα subunit, to which nonphosphorylated STAT-6 is predicted to bind with high affinity. As expected, the *Y606-based peptide inhibits IL-4-induced Tyr phosphorylation of STAT-6 (45). Nevertheless, the inhibition is transient, and evidence that the *Y606-containing peptide had activity in vivo was not presented.

Our data demonstrate that the STAT-6-IP inhibited OVA-induced production of Th2 cytokines from cultured murine splenocytes. Abundant data indicate that IL-4 induces Tyr phosphorylation and activation of STAT-6. Significantly, in the absence of STAT-6, Ag-stimulated murine splenocytes cannot produce IL-4 (15), indicating that a positive feedback loop likely regulates STAT-6 Tyr phosphorylation and IL-4 production in Ag-stimulated murine splenocyte cultures. Based on these data, we hypothesized that inhibition of STAT-6 in murine splenocytes would decrease Th2 cytokine output in response to OVA stimulation. This was indeed the case. The STAT-6-IP inhibited production of both IL-4 and IL-13 in cultured splenocytes, whereas IFN-γ production was unaffected. The STAT-6-IP did not inhibit OVA-induced Tyr phosphorylation of STAT-6 in these cells (data not shown); however, this is not unexpected because the STAT-6-IP, which contains the sequence surrounding *Y641 of STAT-6 itself, is predicted to preferentially inhibit STAT-6 that has been activated, as opposed to inhibiting recruitment of nonactivated STAT-6 to the receptor.

In addition to regulating Th2 cytokine production from cultured splenocytes, STAT-6 also regulates production of eotaxin and other chemokines from epithelial cells (22, 23). Similar to others, we found that eotaxin-3/CCL26 was produced within 24 h of IL-4 stimulation and continued to accumulate for at least 4 days. In STAT-6-IP-treated cells, IL-4-induced accumulation of eotaxin-3/CCL26 was inhibited in a time-dependent manner. The reason for the delay in inhibition is not clear, but we are testing the hypothesis that the STAT-6-IP, over time, acquires the ability to selectively inhibit DNA binding or nuclear translocation of activated STAT-6. As in murine splenocytes, the STAT-6-IP did not inhibit IL-4-induced STAT-6 Tyr phosphorylation in these cells.

In addition to inhibiting cytokine and chemokine production in vitro, when delivered intranasally to the site of OVA challenge in murine models of rhinitis and asthma, the STAT-6-IP effectively inhibited many features of allergic airways disease. The STAT-6-IP inhibited inflammation, IL-13 levels, and AHR when delivered with OVA to the upper airways in a murine model of rhinitis and mild asthma. Moreover, in a moderate to severe asthma model in which inhaled OVA is delivered to the lung, the STAT-6-IP also inhibited mucus production and airway eosinophilia. Our data also demonstrate that the fluorescently labeled STAT-6-IP was present in epithelial cells of the airways and that the STAT-6-IP inhibited OVA-induced expression of eotaxin-1/CCL11, muc2, and muc5ac, all of which are produced by airway epithelial cells in vivo in response to IL-4 and/or IL-13 (46, 47). Our data are consistent with that of Luster and colleagues (12), who demonstrated that lung-specific STAT-6 expression regulates airway inflammation, mucus production, and AHR, possibly through the STAT-6-dependent elaboration of chemokines. In addition, epithelial cell-specific STAT-6 expression may also be sufficient for IL-13-induced AHR in mice (13). Thus, it is likely that inhibition of STAT-6 activity by the STAT-6-IP in airway epithelial cells is at least in part responsible for inhibition of inflammation and AHR in allergic mice. Inhibition of STAT-6 activity in inflammatory cells may also contribute to the ability of the STAT-6-IP to inhibit Th2-dependent allergic responses in these models. For example, inhibition of STAT-6 activity in Ag-specific T cells in the airways may decrease their Th2 cytokine output and thus their ability to induce expression of mucus, cytokines, and chemokines that contribute to and propagate the Th2-dependent inflammatory responses. Future studies will address how STAT-6-IP-dependent inhibition in these different lung compartments contributes to the ability of the STAT-6-IP to inhibit allergic airways disease.

In conclusion, our data show that the novel cell-penetrating STAT-6-IP inhibited STAT-6-dependent chemokine production in vitro in human cells as well as Th2 cytokine production in vitro and in vivo in murine systems. Topical, intranasal delivery of the STAT-6-IP also effectively inhibited many features of allergic airways disease, including airway eosinophilia, mucus production, and AHR. Altogether, these data demonstrate that protein transduction technology can be exploited to inhibit intracellular targets that regulate allergic airways disease. Local application of specific cell-penetrating peptide inhibitors of STAT-6 is a promising new therapeutic approach for the treatment of allergic rhinitis and asthma.

Acknowledgments
We thank Drs. David Eidelman, Brian Ward, and Ron Sullivan for helpful comments regarding the manuscript; Dr. Ron Sullivan for assistance in statistical analyses and Dr. John Dimiao for help with Fig. 1A; and Hector Valderrama-Carvajal and Natalya Karp for help with peptide stability studies and PAS staining, respectively.

Disclosures
The authors have no financial conflict of interest.


