

ORIGINAL ARTICLE Experimental Models of Allergic Disease

Effective treatment of experimental ragweed-induced asthma with STAT-6-IP, a topically delivered cell-penetrating peptide

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Summary

Background Treatment of allergic airways disease including asthma remains primarily local immunosuppression with topical corticosteroid and symptomatic management with antihistamines and anti-leucotrienes. We have developed a novel topical therapy designed to specifically inhibit the events associated with Th2 cell activation.

Objective We assessed the efficacy of our cell-penetrating STAT-6 inhibitory peptide (STAT-6-IP), a novel treatment for allergic airways disease, in a model of chronic ragweed-induced asthma.

Methods Six- to eight-week-old mice were sensitized over 5 weeks with intranasal (IN) exposures to whole ragweed allergen without adjuvant. Mice were then IN challenged with Amba 1 with and without treatment IN with STAT-6-IP and allergic responses assessed. Two weeks later, some animals were rechallenged with Amba 1 with or without STAT-6-IP.

Results Animals exposed to IN ragweed developed significant airway hyperresponsiveness and airways inflammation upon challenge. Cell cultures showed increases in Th2 cytokines IL-4 and IL-13. Topical STAT-6-IP treatment reduced production of Th2 cytokines, demonstrated increased expression of IL-10 and reduced frequency of cultured IL-4 positive CD4⁺ T cells derived from treated mice, suggesting that STAT-6-IP treatment may be immunomodulatory. Airway responsiveness to methacholine challenge in the treatment group was similarly reduced to that of the non-allergic PBS-exposed animals. Importantly, STAT-6-IP-treated mice remained hyporesponsive following second ragweed challenge 2 weeks after treatment.

Conclusions and Clinical Relevance These data suggest that topical application of the STAT-6-IP is sufficient to inhibit allergic airways responses in animals chronically sensitized and challenged with ragweed. Data show that a single topical treatment course is sufficient to block signs of allergic responses to ragweed in the airways for at least 2 weeks. STAT-6-IP is a novel potential treatment for chronic allergic asthma.

Keywords allergic airways, animal model, asthma, immunomodulation, ragweed, STAT-6, treatment

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Introduction

Asthma and allergy are frequent chronic diseases with significant associated morbidity. Allergic asthma is associated with inappropriate immune responses to otherwise innocuous aeroallergens, characterized by the presence of CD4⁺ T cells expressing Th2 cytokines such

as IL-4 and IL-13 and the production of allergen-specific IgE. Conventional treatment modalities, including inhaled and systemic corticosteroids, given after the allergic phenotype is established, only mitigate disease pathogenesis [1]. Attempts to modify development of the allergic phenotype, for example, through prevention of allergen exposure, have been disappointing [2–4].

We have developed an experimental model of chronic ragweed-induced allergic airways disease. Sensitization

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to ragweed was induced through intranasal instillation of allergen over a period of several weeks. After challenge, ragweed-sensitized animals develop significant Th2-mediated airways inflammation and hyperresponsiveness. Using this model, we tested the effects of intranasal application of a chimeric cell-penetrating STAT-6-inhibitory peptide (STAT-6-IP), previously shown to inhibit allergic responses in an acute OVA asthma model [5], to determine if the STAT-6-IP is an effective intervention for chronic ragweed-induced experimental asthma.

The chimeric STAT-6-IP comprises two domains, a protein transduction domain derived from HIV-TAT [6] coupled to eight amino acids surrounding the phosphotyrosine residue at position 641 of STAT-6 [5]. The first domain facilitates cellular uptake of the STAT-6 peptide, probably by endocytosis and the second domain was designed to interfere with the normal functioning of activated STAT-6. The STAT-6 transcription factor is activated by cytokines IL-4 and IL-13 [7] and regulates Th2-dependent gene expression, in part, via up-regulation of GATA3 [8]. Activation of STAT-6 in resident airway cells induces chemokine production, which in turn promotes inflammatory cell influx [9] and STAT-6 activation in airway epithelial cells is required for mucus cell metaplasia/hyperplasia [9,10].

The peptide, STAT-6-IP, is a dominant-negative inhibitor of STAT-6 activity and we have previously demonstrated inhibition of STAT-6-dependent events in acute allergic airway responses to an OVA challenge model of asthma using STAT-6-IP [5]. We were interested in testing the effects of this treatment in a newly developed chronic ragweed-induced asthma model and in evaluating the duration of inhibition. In the present study, we demonstrate that topical STAT-6-IP treatment, given prior to allergen challenge, prevents airway inflammation and hyperresponsiveness. We show that a single course of STAT-6-IP treatment significantly inhibits airway hyperresponsiveness for at least 2 weeks upon subsequent re-challenge. The duration of the inhibition suggests that STAT-6-IP may have potential for long-term airway immunomodulation in allergic individuals.

Methods

Animals

Six- to eight-week-old BalB/c mice were obtained from Harlan-Sprague Dawley (Indianapolis, IN, USA) and were housed in a conventional animal facility at the Meakins-Christie Laboratories. For each experimental condition, a minimum of six to eight animals were used. All studies followed CCAC guidelines and were approved by the Animal Care Committee at McGill University.

Ragweed sensitization and challenge

Awake animals were sensitized 5 days per week for 5 weeks by local instillation of 10 µL in each nare of 1% (10 mg/mL) ragweed (Giant Ragweed, extracted from *Ambrosia trifida*, Sigma-Aldrich, St Louis, MO, USA) in PBS. After a 2-week rest, mice were challenged with 1% (10 mg/mL, dry weight) Amba I (Short Ragweed, extracted from *ambrosia artemisiifolia* pollen grains; Greer, Lenoir, NC, USA) in PBS once per day for five consecutive days and assayed 24 h following the final challenge. In some experiments, mice were briefly anaesthetized with isoflurane prior to ragweed challenges. Animals treated with peptides received the STAT-6-IP (150 µg) or control STAT-6-CP (150 µg) in 10 µL PBS intranasally prior to each ragweed challenge (Fig. 1a). To assess the duration of the inhibitory effect of the STAT-6-IP, following the first series of ragweed challenges, mice were given a 2-week 'rest' and subsequently re-challenged with or without the STAT-6-IP (see Fig. 5).

Peptide synthesis

STAT-6-inhibitory peptides (STAT-6-IP or IP) and STAT-6-control peptides (STAT-6-CP or CP) were synthesized by the University of Calgary Integrated Peptide Services. Peptides comprise a derivative of the TAT protein transduction domain, YARAAARQARA [6]. This sequence is coupled to an 8 amino acid sequence surrounding tyrosine 641 in murine STAT-6. In the STAT-6-IP, the tyrosine residue is phosphorylated (GRG*YVSTT) and in the control STAT-6-CP peptide, this residue is replaced by a phenylalanine (GRGFVSTT). Peptides were amidated at the carboxyl terminus and purified by RP-HPLC and analysed by MS. Peptide sequences, uptake, stability *in vivo* and inhibitory effects of the STAT-6-IP have been detailed previously in an acute OVA-induced allergic airways disease model [5].

Assessment of airway hyperresponsiveness by methacholine challenge

Bronchial responsiveness was measured 24 h after ragweed challenge using a small animal ventilator (FlexiVent; SCIREQ, Montreal, QC, Canada) as described elsewhere [5]. Briefly, mice were deeply anaesthetized with xylazine hydrochloride (NovaPharm Limited, Toronto, ON, Canada) followed by sodium pentobarbital (CEVA, Santé Animale, France) and paralyzed with pancuronium bromide. Heart rate was monitored by EKG to ensure adequate anaesthesia throughout the procedure. Mice were ventilated quasi-sinusoidally with a tidal volume of 8 mL/kg and respiratory rate of 150 breaths/min. Methacholine was given by nebulizer (Aeroneb; SCIREQ, Montreal, QC, USA) at doses of 3.1, 12.5, and

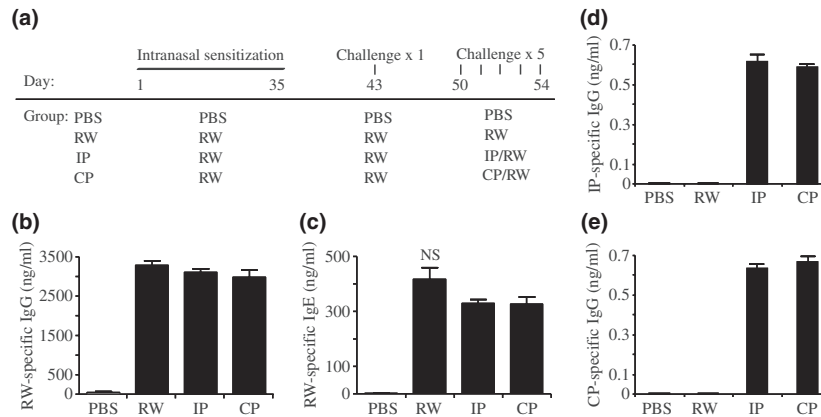


Fig. 1. Ragweed (RW) induces both IgG and IgE-specific antibody responses in experimental allergic airways while peptide treatment is poorly immunogenic. (a) Sensitization and challenge protocol of chronic allergic airways disease induced by ragweed allergen and timing of STAT-6-IP treatment. Mice were intranasally (IN) sensitized and challenged with PBS or RW. Before each challenge, some animals were IN treated with 150 μ g of STAT-6-IP (IP) or STAT-6-CP (CP). Ragweed-specific antibodies, IgG (b) and IgE (c), in sera were quantified by ELISA. STAT-6-IP (d) and CP (e) peptides elicited only weak IgG responses (5000-fold less than that for ragweed). No peptide-specific IgE was detected (data not shown) (minimum of six to eight animals per group; mean $\pm$ SEM is shown).

50 mg/mL and maximal resistance was obtained for each graded dose of methacholine.

Measurement of ragweed-specific IgG and IgE

ELISA plates were coated with 20 mg/mL short ragweed. For assessment of ragweed-specific IgG, standard purified mouse IgG (Zymed Laboratories, San Francisco, CA, USA) was used and serially diluted. Appropriate dilutions of serum samples were loaded into each well and incubated for 2 h at 37°C. Plates were washed and biotin-labelled rabbit anti-mouse IgG plus HRP-streptavidin (Zymed Laboratories) was added. Following washing, TMB substrate (Pharmingen OptEIA™, San Diego, CA, USA) was added and the reaction was stopped with 1 M H₃PO₄ and read at 450 nm. Ragweed-specific IgE was performed using an ELISA kit (Pharmingen OptEIA™ IgE Kit) with the following modifications: serum was incubated overnight at 4°C with protein G sepharose beads (Amersham Bioscience, Piscataway, NJ, USA) prior to loading onto ELISA plates that had been previously coated with short ragweed. Standards were plated as per OptEIA protocol. Samples were incubated overnight at 4°C and ELISA was performed as per protocol. For peptide-specific IgG or IgE, the plates were coated with 100 μ L of 2 μ g/mL of either STAT-6-IP or STAT-6-CP peptide, respectively. The specific antibodies were quantified with mouse IgG or IgE ELISA sets (BD Biosciences Pharmingen, San Diego, CA, USA).

Bronchoalveolar lavage (BAL)

Immediately post-mortem, lungs were lavaged twice *in situ* with 0.75 mL of ice-cold saline. The returned fractions were pooled and centrifuged. The supernatants

from the first returned fractions were stored at -80°C until assayed for cytokines. The pellets were used for total and differential cell counts as previously described [5]. Briefly, red blood cells were lysed using ACK lysing buffer (PharM Lyse™; BD Biosciences, Mississauga, ON, Canada). Cells were spun onto glass slides and stained using the Diffquick method. Differential cell counts were obtained manually under light microscopy. 2–3 $\times$ 100 cells were counted per slide and means obtained.

RNA extraction and real-time RT-PCR

Whole lung RNA levels encoding muc5ac, muc2 and eotaxin-1/CCL11 were assessed by Real-time RT-PCR. Total cellular RNA was extracted from the lung using an RNeasy Mini Kit (Qiagen, Mississauga, ON, Canada), and complementary DNA was prepared using SuperScript II reverse transcriptase (Invitrogen, Burlington, ON, Canada), according to the manufacturer's protocols. The targets were amplified using specific primers as detailed in supplementary Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as endogenous reference gene for relative quantification. PCR was performed with a LightCycler System (Roche Applied Sciences, Laval, QC, Canada) using QuantiTect SYBR Green (Qiagen), with cycling parameters of 95°C for 15 min followed by 45 cycles of 95°C for 15 s, 63°C for 20 s and 72°C for 20 s. All samples were normalized to the corresponding reference gene, and the results were calculated according to individual internal standards.

Cell culture and cytokine assessment by ELISA

Single-cell suspensions were prepared from whole spleen. Erythrocytes were lysed by ACK lysing buffer

and the washed splenocytes were resuspended at 5×10^6 cells/mL in complete RPMI-1640 medium with 10% heat-inactivated FCS, 2 mM L-glutamine, 50 μ M 2-ME, 100 U/mL penicillin, 100 μ g/mL streptomycin sulphate. Splenocytes were then cultured for 4 days at 37°C in the presence of 100 μ g/mL Amba I. Supernatants were stored at -80°C prior to quantifying cytokine levels by ELISA (see below). Murine IL-4, IL-5, IL-10, IL-13 and IFN- γ levels in supernatants of the ragweed-stimulated cultured splenocytes and BAL fluid were quantified using ELISA kits purchased from eBioscience Inc (San Diego, CA, USA) according to the manufacturer's instructions.

Flow cytometry

The frequency of IL-4 and IFN- γ expressing CD4⁺ T cells was evaluated by flow cytometry. Splenocytes that had been cultured for 4 days with Amba I were stimulated for 5 h with murine anti-CD3 (0.5 μ g/mL; clone 2C11, BD Pharmingen) and monensin (GolgiStop; BD Biosciences), according to the manufacturer's instructions. Cells were then washed, permeabilized with saponin (Perm/Wash; BD Biosciences), and fixed in formaldehyde and PBS (Cytofix/Cytoperm; BD Biosciences) for 30 min. Splenocytes were then stained with FITC-conjugated rat anti-mouse CD4, PE-conjugated rat anti-mouse IL-4, and APC-conjugated rat anti-mouse IFN- γ (all from BD Biosciences). Data were acquired using a FACSCalibur (Becton Dickinson, San Jose, CA, USA). Intracellular IL-4 and IFN- γ were analysed by gating on CD4-positive cells using CellQuest software (Becton Dickinson).

Histological Analysis

Histology was performed as described previously [5]. Briefly, lungs were slowly inflated with 1 mL of formalin and then placed entirely in formalin. The skull was also isolated and placed in formalin and decalcified with 4% EDTA-Na for 4 weeks. The specimens were embedded in paraffin and 0.5 μ m sections were cut. Slides were stained with periodic acid Schiff (PAS) to assess mucus under standard light microscopy.

Statistical analysis

The results are expressed as mean $\pm$ SEM. Statistical significance was measured by one-way ANOVA followed by Tukey's *posthoc* tests for individual group comparisons using SPSS software (Studica Limited, Mississauga, ON, Canada). Group comparisons as well as individual comparisons for each data set are reported in the figure legends. A value of $P < 0.05$ was considered significant.

Results

Intranasal administration of ragweed induces specific IgE

Intranasal administration of ragweed induced both IgG and IgE specific for the ragweed allergen (Figs 1b and c). Intranasal administration of the STAT-6-IP prior to each of 5 daily challenges had no effect on IgE or IgG levels. In addition, peptide-specific antibodies were detected only at extremely low levels in animals exposed to multiple doses of intranasal peptide (Fig. 1d), suggesting that the peptide alone is poorly immunogenic.

STAT-6-IP treatment during challenge inhibits Th2 activity

Splenocytes, from ragweed allergic animals, treated *in vivo* with or without STAT-6-IP during the challenge phase, were cultured for 4 days with Amba 1 and cytokine levels assayed. While levels of IL-13, IL-4, and IL-5 were significantly reduced in cultures from STAT-6-IP-treated animals, IFN- γ levels did not change, regardless of treatment group (Figs. 2a–d). IL-10 levels were significantly increased following STAT-6-IP treatment (Fig. 2e). Importantly, the frequency of IL-4-positive cells was also significantly reduced in STAT-6-IP-treated animals, providing evidence that STAT-6-IP treatment *in vivo* resulted in a decreased expansion of IL-4 positive cells (Figs. 2f and g).

STAT-6-IP treatment inhibited ragweed-induced airway hyperresponsiveness to levels that did not differ from those in the saline-treated group. BAL fluid numbers of total inflammatory cells as well as eosinophils were significantly reduced in STAT-6-IP-treated mice compared with ragweed-allergic controls (Figs 3a and b). STAT-6-CP treatment showed no effect. In addition, cytokine levels in BAL fluid showed increases in IL-13, IL-4 and IL-5 in ragweed-allergic animals and in those treated with the STAT-6-CP control peptide (Fig. 3). These levels were significantly reduced in the STAT-6-IP treated animals, suggesting that STAT-6-dependent cytokine-mediated responses to ragweed challenge were inhibited. IL-10 levels were significantly increased in BAL from the STAT-6-IP treated mice (Fig. 3g). Finally, we examined both upper and lower airways for evidence of inflammation and mucous production. Ragweed-allergic animals developed marked airway inflammation and mucous cell hyperplasia (Figs 4a and b) that were both effectively inhibited upon treatment with the STAT-6-IP, but not with the STAT-6-CP. As well, lung levels of mRNA encoding MUC5A, MUC2 and eotaxin/CCL11 were up-regulated in the ragweed and STAT-6-CP-treated animals (Fig. 4).

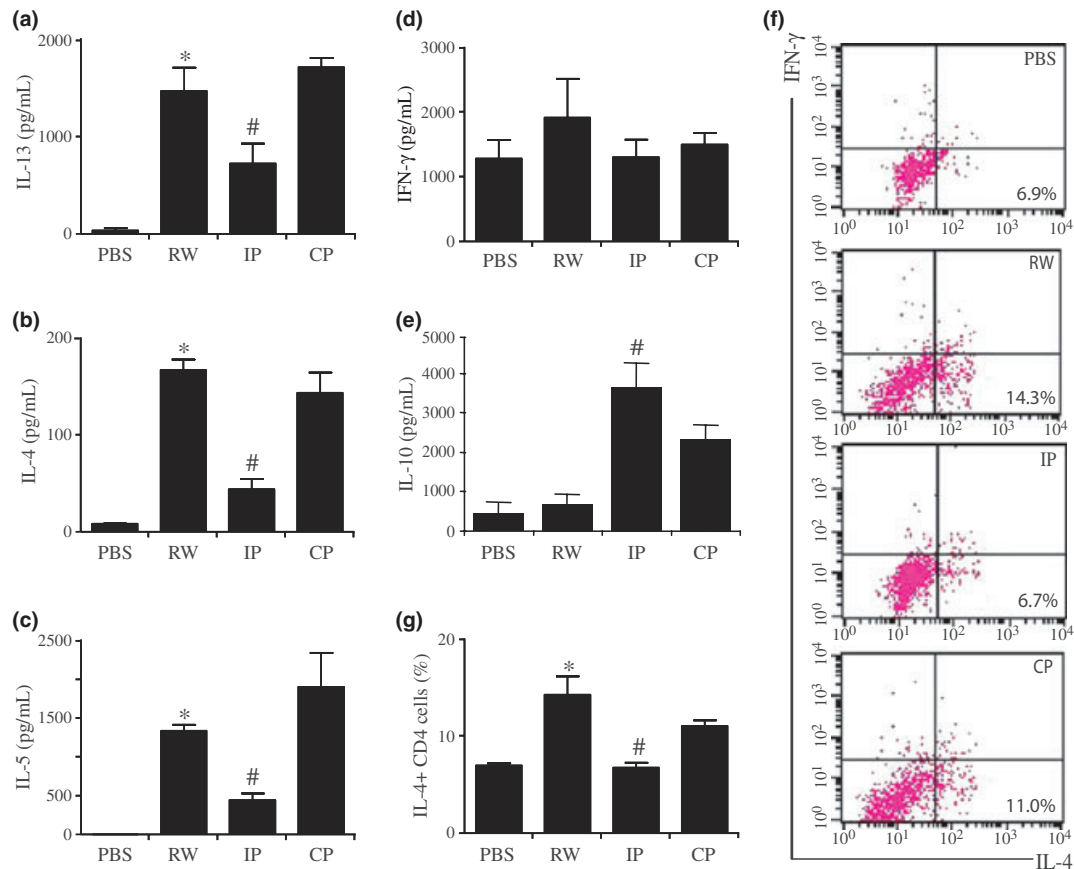


Fig. 2. Th2 cytokine expression and the frequency of IL4 positive cells are significantly decreased in splenocyte cultures from IP-treated ragweed-allergic mice. Total splenocytes, from allergic and control animals treated with or without STAT-6-IP, were cultured with Amba 1 for 4 days and cytokine levels measured in the supernatants by ELISA. IL13 (a), IL4 (b) and IL5 (c) levels were significantly decreased in IP-treated mice compared with controls. IFN- γ levels were unchanged (d) while IL-10 levels (e) were significantly increased in STAT-6-IP treated mice. Mean + SEM values of three independent experiments with six to eight animals per group are reported (f) Intracellular cytokine staining for IL-4 and IFN- γ in CD4⁺ T cells was assessed by flow cytometry. (g) Percentage of CD4⁺ T cells expressing IL-4 was quantified, and mean $\pm$ SEM value from three independent experiments are reported. * P < 0.01 vs. PBS group; # P < 0.01 vs. CP group.

Expression of each of these genes was reduced in STAT-6-IP-treated mice. Altogether, these data suggest that STAT-6-IP treatment in ragweed-allergic asthmatic mice prevented allergic inflammation and Th2 cell activity upon subsequent exposure to ragweed.

Duration of Effect

We next examined the duration of peptide inhibitory effects. Sensitized animals were challenged with ragweed alone or with ragweed plus the STAT-6-IP (or STAT-6-CP). After a 2-week rest, animals were again challenged with ragweed alone or with ragweed plus the STAT-6-IP (Fig. 5a). Animals receiving STAT-6-IP prior to both ragweed challenge phases showed no evidence of airway hyperresponsiveness (Fig. 5b:2xIP group). Importantly, compared with ragweed-allergic animals, mice given the STAT-6-IP only during the first series of challenges also showed significantly reduced

airway hyperresponsiveness even after a second series of challenges. Cell differential counts from BAL fluid demonstrate persistent reduction in total cell number and a significant reduction in eosinophils in STAT-IP-treated animals, even those treated only at first challenge, compared with ragweed control group (Fig. 5c). In addition, cultured splenocytes from animals treated with the STAT-6-IP only during the first challenge phase demonstrated reduced production of IL-13, IL-4 and IL-5, to levels that did not differ from those in mice treated with STAT-6-IP before both challenges (Figs 5d–f). There were no differences in IFN- γ levels between the groups (Fig. 5g). Finally, IL-10 levels were consistently increased in animals treated with STAT-6-IP before only the first or at both challenge phases (Fig. 5h). These data suggest that STAT-6-IP treatment has a prolonged duration of effect, which may represent important immunomodulation from the established Th2-type to a regulatory-type response.

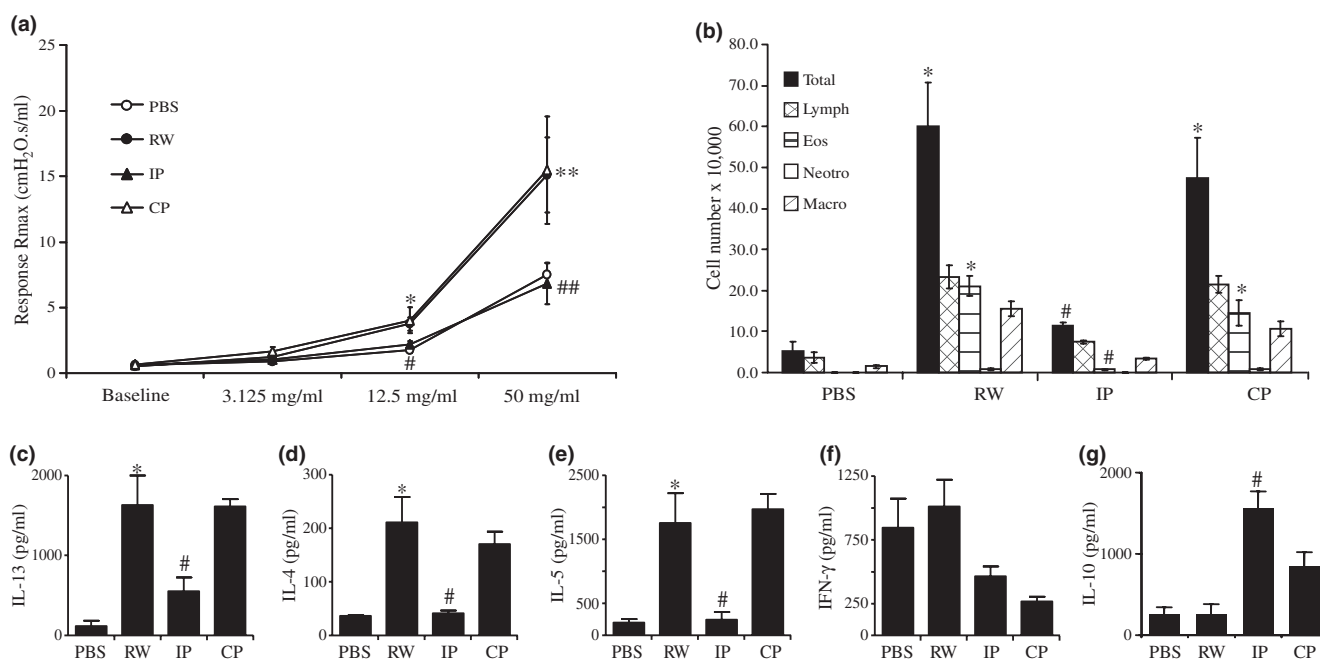


Fig. 3. Ragweed-induced airway hyperresponsiveness, influx of inflammatory cells and Th2 cytokine levels are significantly inhibited by STAT-6-IP treatment. (a) Ragweed-allergic animals show marked increases in mean airway resistance ($\pm$ SEM) following aerosol methacholine challenge, which is abrogated to baseline levels by STAT-6-IP treatment. * $P < 0.05$ vs. PBS group; ** $P < 0.01$ vs. PBS group; ## $P < 0.01$ vs. CP group. Five to eight animals per group are reported. (b) Differential cell counts from bronchoalveolar lavage fluid (BAL) show reduction in total cell counts and total and relative eosinophils in STAT-6-IP-treated animals. Mean $\pm$ SEM of six to eight animals per group are reported. * $P < 0.01$ vs. PBS group; # $P < 0.01$ vs. RW group.

Discussion

In this study, we show that STAT-6-IP pre-challenge treatment effectively inhibited the allergic response in the airways of animals chronically exposed to ragweed. Effects of treatment include reductions in mucus production, airway inflammation, as well as AHR. We show that this inhibition was associated with a reduction in the frequency of IL-4-positive cells following challenge and that STAT-6-IP treatment resulted in persistent inhibition, despite repeated ragweed challenge, that was demonstrable for at least 2 weeks.

We have previously shown inhibition of experimental allergic asthma by the STAT-6-IP in an acute OVA-induced model [5]. In the present study, we have developed and characterized a novel model of ragweed allergen-induced allergic airways disease. In this model, mice were sensitized and challenged exclusively via the intranasal route, without the use of adjuvants. Unlike other published models [11–19], many of which sensitize by administration of ragweed plus adjuvant via the intraperitoneal route, we extended the sensitization period to 5 weeks of intranasal allergen exposure (5 days per week) to mimic as closely as possible the route and extent of sensitization in humans. Previous studies have shown that ragweed sensitization can be

achieved by intranasal exposure in a shorter period of time if the environment is enriched with GM-CSF [11] or if multiple allergens are used in a chronic model [13]. Our data demonstrate significant reproducible airways inflammation upon subsequent allergen exposure and recapitulate the same response on re-challenge using ragweed alone, in the absence of other cytokines or modifiers such as adjuvants. (Figs 3 and 5). These data suggest that the intranasal route of allergen exposure, without additional adjuvant, is sufficient to induce Th2 responses and experimental allergic asthma. In addition, in our chronic model, animals showed persistence of a robust Th2 allergic response for at least 70 days after first exposure, suggesting that prolonged monosensitization results in established Th2-mediated allergic lower airway responses that persist after multiple challenges.

Using this model, we evaluated the effects of STAT-6-IP treatment in both challenge and re-challenge protocols. STAT-6-IP treatment inhibited Th2-biased airway inflammatory responses when administered prior to each of 5 daily allergen challenges. Significantly, STAT-6-IP-treated animals were largely resistant to a second course of allergen challenge after a 2-week interval. We have previously shown that the STAT-6-IP has a limited half-life (4 h) and studies of other

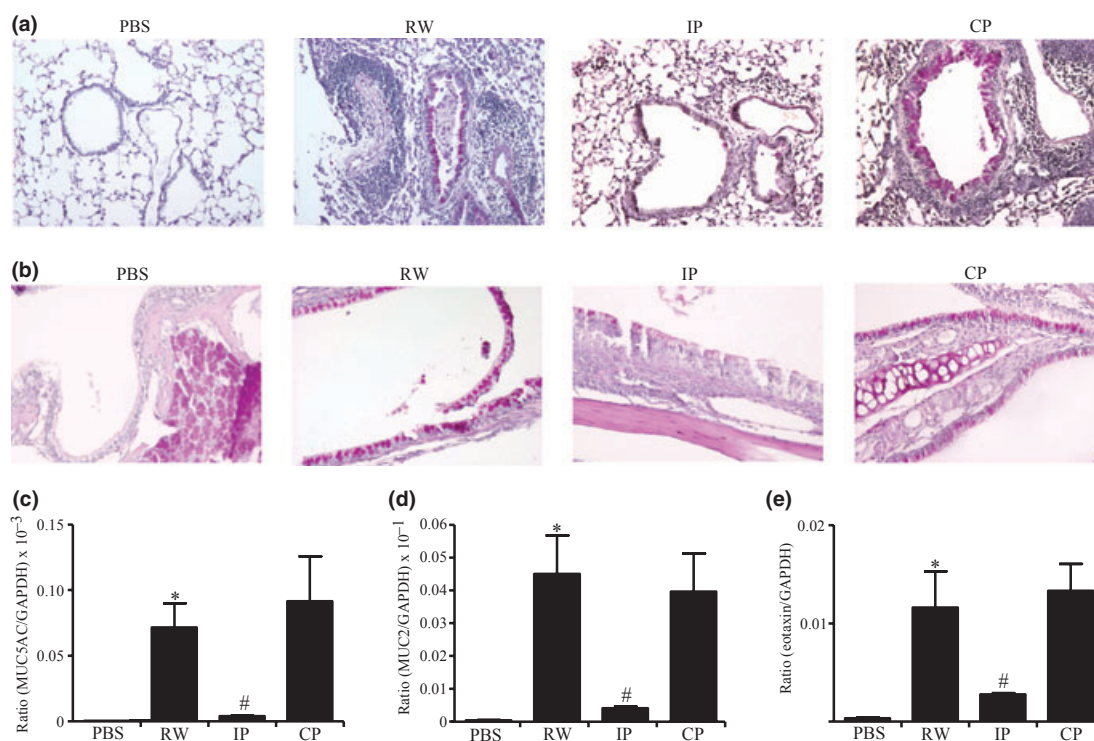


Fig. 4. Ragweed challenge induces increase in airway inflammation and mucous production, which is inhibited by STAT-6-IP treatment. Lung (a) and nasal (b) tissues were harvested and embedded in paraffin, and mucus-positive cells were identified by staining sections with PAS (magnification 200x). RNA was extracted from whole lung homogenates and expression of MUC5A (c), MUC2 (d) and eotaxin-1 (e) were quantified by real-time RT-PCR. Values were reported compared with the GAPDH reference gene expression. * $P < 0.01$ vs. PBS group; # $P < 0.01$ vs. RW or CP group.

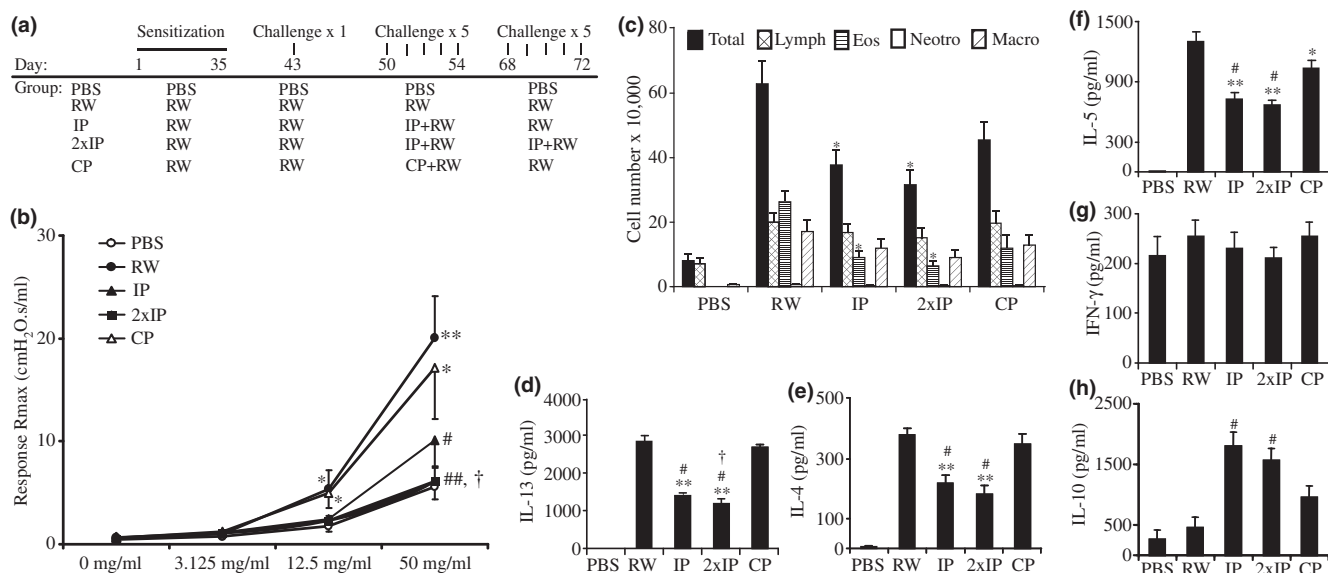


Fig. 5. STAT-6-IP treatment induces prolonged inhibition of ragweed-induced experimental asthma on re-challenge. Two weeks after the challenge phase, ragweed-sensitized mice were re-challenged with and without peptide (a). The mean airway resistance following an aerosol methacholine challenge shows reduction to baseline in animals treated prior to both or only the first challenge with STAT-6-IP (b). * $P < 0.05$ vs. PBS group; ** $P < 0.01$ vs. PBS group; # $P < 0.05$ vs. RW group; ## $P < 0.01$ vs. RW; † $P < 0.05$ vs. CP group. Differential cell counts from BAL show reduction in total cell counts and total and relative eosinophils in mice treated with STAT-6-IP prior to both or only the first challenge (c). Mean values of 6 animals per group $\pm$ SEM. * $P < 0.05$ vs. RW group. Total splenocyte cultures from each group were assayed for cytokine expression by ELISA (d–h). Mean values of six individual mice were reported $\pm$ SEM. * $P < 0.05$ vs. RW group; ** $P < 0.01$ vs. RW group; # $P < 0.01$ vs. CP group; † $P < 0.05$ vs. IP group.

cell-penetrating peptides show rapid clearance of these molecules from the circulation [20]. Thus, we expected that animals re-challenged 2 weeks after the initial STAT-6-IP treatment would have robust ragweed-induced inflammation and airway hyperresponsiveness. To our surprise, STAT-6-IP-treated mice were refractory to subsequent allergen challenge 2 weeks later. We have shown that *in vivo* STAT-6-IP treatment reduces frequency of IL-4 + ve cells in splenocyte cultures (Figs 2f and g) and up-regulates IL-10 expression (Figs 2e, 3g and 5g). Taken together, these data suggest that the STAT-6-IP not only inhibited STAT-6-dependent events leading to allergic inflammation and reactive airways but also inhibited expansion of IL-4-expressing cells. Our data showing prolonged protection from allergic inflammation (Figs 5b and c) associated with increased IL-10 expression (Fig. 5h) support the hypothesis that transient STAT-6 inhibition in the airway is immunomodulatory and may result in immuno-deviation inducing the prolonged refractory period following treatment, findings which enhance the translational potential of STAT-6-IP treatment to the clinical setting. Experiments are currently underway to determine the effects of STAT-6 inhibition in repeatedly challenged mice with and without IL10 blockade. Data from these experiments may help determine the role, if any, of IL-10 release in STAT-6-IP-mediated inhibition.

The feasibility of STAT-6-IP use for treatment of human asthma relies on several factors. Importantly, STAT-6-IP is poorly immunogenic itself (Figs 1d and e). The potential for clinical application of cell-penetrating peptide-based therapies is being investigated for many different diseases including cancer [21], brain injury [22] and stem-cell modification prior to transplant [23]. Limitations include the difficulty in targeting specific

cells (or cell types) and rapid clearance from circulation [20,24]. Our data demonstrate that the STAT-6-IP can be delivered intranasally, ensuring efficient delivery to the local airways and these data show that this mode of delivery is efficient and sufficient for inhibition of airway hyperresponsiveness and inflammation. We also show reduced Th2 cytokines in allergen-cultured splenocytes from animals treated with STAT-6-IP intranasally suggesting that cells affected by this treatment circulate to other lymphoid organs. Medoff *et al.* [9] have shown that STAT-6 activity in myeloid cells is required for chemokine production and recruitment of inflammatory cells and Th2 cells into the airways in allergic asthma. We suggest that STAT-6-IP may act, directly and/or *in trans*, on myeloid cells in the airway. We are presently exploring the effects of *in vitro* STAT-6-IP treatment of dendritic cells with the hypothesis that DC-specific STAT-6 inhibition may be sufficient to inhibit the allergic phenotype in established disease. If true, other routes of peptide delivery, such as sublingual or subcutaneous, may prove to be effective. Finally, the finding that STAT-6-IP treatment has a protracted duration of effects on allergic airway responses enhances the potential of this novel therapy for long-term control of asthma in humans.

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References

- 1 Barnes PJ. New therapies for asthma: is there any progress? *Trends Pharmacol Sci* 2010; 31:335–43.
- 2 Chan-Yeung M, Ferguson A, Watson W *et al.* The Canadian Childhood Asthma Primary Prevention Study: outcomes at 7 years of age. *J Allergy Clin Immunol* 2005; 116:49–55.
- 3 van Schayck OC, Maas T, Kaper J, Knottnerus AJ, Sheikh A. Is there any role for allergen avoidance in the primary prevention of childhood asthma? *J Allergy Clin Immunol* 2007; 119: 1323–8.
- 4 Arshad SH, Bateman B, Sadeghnejad A, Gant C, Matthews SM. Prevention of allergic disease during childhood by allergen avoidance: the Isle of Wight prevention study. *J Allergy Clin Immunol* 2007; 119:307–13.
- 5 McCusker CT, Wang Y, Shan J *et al.* Inhibition of experimental allergic airways disease by local application of a cell-penetrating dominant-negative STAT-6 peptide. *J Immunol* 2007; 179:2556–64.
- 6 Ho A, Schwarze SR, Mermelstein SJ, Waksman G, Dowdy SF. Synthetic protein transduction domains: enhanced transduction potential *in vitro* and *in vivo*. *Cancer Res* 2001; 61:474–7.
- 7 Hershey GKK. IL-13 receptors and signaling pathways: an evolving web. *J Allergy Clin Immunol* 2003; 111:677–90.
- 8 Zheng W, Flavell RA. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell* 1997; 89:587–96.
- 9 Medoff BD, Seung E, Hong S *et al.* CD11b+ myeloid cells are the key mediators of Th2 cell homing into the airway in allergic inflammation. *J Immunol* 2009; 182:623–35.
- 10 Kuperman DA, Huang X, Koth LL *et al.* Direct effects of interleukin-13 on epithelial cells cause airway hyperactivity and mucus overproduction in asthma. *Nat Med* 2002; 8:885–9.
- 11 Cates EC, Gajewska BU, Goncharova S *et al.* Effect of GM-CSF on immune, inflammatory, and clinical responses to ragweed in a novel mouse model of

- mucosal sensitization. *J Allergy Clin Immunol* 2003; **111**:1076–86.
- 12 Dharajiya N, Vaidya S, Sinha M, Luxon B, Boldogh I, Sur S. Allergen challenge induces Ifng dependent GTPases in the lungs as part of a Th1 transcriptome response in a murine model of allergic asthma. *PLoS ONE* 2009; **4**:e8172.
 - 13 Goplen N, Karim MZ, Liang Q *et al.* Combined sensitization of mice to extracts of dust mite, ragweed, and *Aspergillus* species breaks through tolerance and establishes chronic features of asthma. *J Allergy Clin Immunol* 2009; **123**: 925–32 e11.
 - 14 Kruzel ML, Bacsí A, Choudhury B, Sur S, Boldogh I. Lactoferrin decreases pollen antigen-induced allergic airway inflammation in a murine model of asthma. *Immunology* 2006; **119**:159–66.
 - 15 Mishra NC, Rir-Sima-Ah J, Langley RJ *et al.* Nicotine primarily suppresses lung Th2 but not goblet cell and muscle cell responses to allergens. *J Immunol* 2008; **180**:7655–63.
 - 16 Nadeem A, Fan M, Ansari HR, Ledent C, Jamal Mustafa S. Enhanced airway reactivity and inflammation in A2A adenosine receptor-deficient allergic mice. *Am J Physiol Lung Cell Mol Physiol* 2007; **292**:L1335–44.
 - 17 Nemeth K, Keane-Myers A, Brown JM *et al.* Bone marrow stromal cells use TGF-beta to suppress allergic responses in a mouse model of ragweed-induced asthma. *Proc Natl Acad Sci U S A* 2010; **107**:5652–7.
 - 18 Ponnoth DS, Nadeem A, Mustafa SJ. Adenosine-mediated alteration of vascular reactivity and inflammation in a murine model of asthma. *Am J Physiol Heart Circ Physiol* 2008; **294**: H2158–65.
 - 19 Ponnoth DS, Nadeem A, Tilley S, Mustafa SJ. Involvement of A1 adenosine receptors in altered vascular responses and inflammation in an allergic mouse model of asthma. *Am J Physiol Heart Circ Physiol* 2010; **299**:H81–7.
 - 20 Sarko D, Beijer B, Boy RG *et al.* The pharmacokinetics of cell-penetrating peptides. *Mol Pharm* 2010; **7**: 2224–31.
 - 21 Liu M, Li C, Pazgier M *et al.* D-peptide inhibitors of the p53-MDM2 interaction for targeted molecular therapy of malignant neoplasms. *Proc Natl Acad Sci U S A* 2010; **107**:14321–6.
 - 22 Wang X, Han W, Du X *et al.* Neuroprotective effect of Bax-inhibiting peptide on neonatal brain injury. *Stroke* 2010; **41**:2050–5.
 - 23 Yukawa H, Noguchi H, Nakase I *et al.* Transduction of cell-penetrating peptides into induced pluripotent stem cells. *Cell Transplant* 2010; **19**: 901–9.
 - 24 Jarver P, Mager I, Langel U. In vivo biodistribution and efficacy of peptide mediated delivery. *Trends Pharmacol Sci* 2010; **31**:528–35.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Primers for PCR

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Supplementary Table 1

Primers for PCR

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