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Peptide induces $CD4^+CD25^+$ and IL-10^+ T cells and protection in airway allergy models

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Abstract

The purpose of this study was to evaluate whether a single peptide containing a major T cell epitope might induce peripheral tolerance in a complex allergen model. C57BL/6 mice were sensitized by intraperitoneal injection of house dust mite extract (HDM), and exposed to antigen via trachea instillation. Der p 1 peptide was administered by i.v. before or after sensitization. Lung lavage fluids were analyzed for cellular infiltration. Respiratory exposure of sensitized mice to antigen results in airway inflammation and eosinophilia. Intravenous administration of a single peptide protected sensitized mice from these changes. Further, the emergence of antigen-specific $CD25^+CD4^+$ and IL-10 secreting cell populations in DO11.10 mice was demonstrated after peptide administration. Thus, intravenous delivery of a single peptide epitope is capable of inducing peripheral tolerance and protection in a complex allergy model, possibly through regulatory T cells and bystander suppression.

Keywords: Bystander suppression; House dust mite allergy; Peptide vaccine; Peripheral tolerance; Regulatory T cells

1. Introduction

Allergic asthma is marked by airway hyperresponsiveness (AHR) and inflammation, excess mucus secretion and elevated serum IgE levels. $CD4^+$ Th2 lymphocytes in the airways of allergic individuals and cytokines (IL-4, IL-5, IL-13, etc.) secreted by these cells are thought to be important in the establishment and progression of allergic asthma [1]. IL-4 enhances the immunoglobulin class switch to IgE, regulates the expansion of IgE-dedicated B cells and the production of mast cells. IL-13 is linked to excessive mucus secretion in the airway. IL-5 promotes the differentiation, maturation and activation of eosinophils, as well as functioning as an eosinophil chemotractant. The accumulation of mast cells and eosinophils in the airways following chronic allergen exposure results in the pathology associated with the disease.

Specific immunotherapy (SIT) for allergic disease involves the subcutaneous injection of increasing doses of whole allergen extracts. The use of SIT has been limited due to the risk of anaphylactic reactions triggered by vaccine-induced cross-linking of IgE antibodies anchored on the surface of mast cells. Safer and more effective alternative therapeutic approaches are needed to combat the ever-increasing rate of allergic disorders. One of these approaches involves induction of peripheral tolerance by systemic or mucosal delivery of short synthetic MHC class II binding peptides that contain T helper cell epitopes [2–6]. Peptide-based allergy vaccines may be safer because they do not contain IgE-binding epitopes. However, designing an effective peptide vaccine for human immunization is complicated by the polymorphism of the human MHC molecules and the antigenic complexity of allergens. Often atopic individuals are allergic to multiple antigen sources and each allergen source may have multiple components. There are many types of MHC molecules and each recognizes peptides of different amino acid sequence. For this reason, recent studies have used overlapping peptides spanning large portions of the allergen molecule to immunize

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humans [6–8]. It is not practical for a peptide-based allergy vaccine to encompass all T cell epitopes matching each MHC type. The aim of this study was to determine if immunization with a single peptide is sufficient: (1) to induce tolerance and protection against multi-component allergens in a mouse strain of appropriate MHC background and (2) to determine the immune mechanism of protection. This information has important implications for the rational design of peptide vaccines.

2. Materials and methods

2.1. Allergens and formulations

Whole body *Dermatophagoides pteronyssinus* extract (house dust mite (HDM)) was purchased from Greer Laboratories (Lenoir, NC). Endotoxin-free ovalbumin (OVA) (ICN, Costa Mesa, CA) was used as a model allergen. Der p 1 peptide 114–128 (p114–128) (SNYCQIYPPNANKIR) and OVA peptide 323–339 (p323–339) (ISQAVHAAHAEINEAGR) were synthesized by Biosource International (Hopkinton, MA) with a free carboxyl terminus and HPLC purified. Aluminum hydroxide (alum) was purchased from Accurate Chemical (Westbury, NY).

2.2. HDM allergy models

Six- to eight-week-old female C57BL/6 mice (Charles River, Wilmington, MA) were used. The murine allergy model involved a sensitization and a challenge step. For sensitization, each mouse was injected intraperitoneally (i.p.) with 10–50 μg HDM adsorbed to 1–4 mg alum in 0.5 ml volume using a 26-gauge needle once or twice at weekly intervals. Mice were challenged by spraying intratracheally (i.t.) a saline solution containing 10 μg of HDM into the lungs. Briefly, mice were anesthetized by i.p. injection of ketamine (1.5 mg) and xylazine (0.36 mg). A blunt-end lavage needle attached to a pump-operated Hamilton syringe via a 12-cm long Tygon tubing (80 mm inside diameter) was used for spraying the challenge solution. By pressing the pump, 10 μl solution was dispensed into the lungs as a fine aerosol mist via the lavage needle placed at the opening of the trachea. Each mouse received five sprays, for a total of 50 μl solution containing 10 μg of HDM total protein, to ensure uniform distribution of the challenge solution throughout the lungs. Unless otherwise noted, the challenge was repeated 48 h later and the mice were euthanized with CO_2 after an additional 24 h. Bronchoalveolar lavage fluids (BALF) were collected for differential cell counts and cytokine analysis, and the lungs were collected for histological examination. Blood was collected prior to challenge via retro-orbital bleeding. Unless otherwise noted, eight mice per group were used.

For tolerance induction, mice were injected i.v. via the tail vein with 5 μg p114–128 or p323–339 dissolved in 0.2 ml sterile saline using a 30-gauge needle. In the prophylactic model, the injections were given three times at weekly intervals and were completed 1 week before sensitization. In the therapeutic model, mice received one, two or three injections at varying times after sensitization.

Mice were cared for and maintained under the "Guide for Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press (revised 1996).

2.3. OVA transgenic model

DO11.10 TCR transgenic mice, mated on the Balb/c background, were purchased from Jackson Laboratory (Bar Harbor, ME) and used to study the T cell responses to peptide immunization. The DO11.10 TCR line is transgenic for a receptor that recognizes the OVA peptide 323–339 in the context of I-A^d MHC molecules. The monoclonal antibody, KJ1-26, is specific for the DO11.10 transgenic TCR. Mice were either injected i.v. with 5 μg p323–339 on days 0, 7 and 14, or sensitized by i.p. injection of 10 μg OVA adsorbed to 0.5 mg alum on days 0 and 14.

2.4. Intracellular cytokine and T cell marker staining

Spleens of transgenic mice were harvested on Day 21 and 1×10^6 splenocytes per well of 96-well flat-bottom plates (Falcon BD, Franklin Lakes, NJ) were stimulated with 10 μg/ml OVA in complete RPMI-1640 containing 10% fetal bovine serum (FBS) (Harlan Bioproducts, Indianapolis, IN) and 1:16,000 dilution GolgiStop (BD Biosciences, San Diego, CA) at 37 °C for 24 h. Control wells contained medium alone. Cells were then collected, transferred to 96-well round-bottom plates (Falcon BD) and incubated on ice for 30 min with Cy-Chrome-labeled anti-CD4, allophycocyanin-labeled anti-CD25 and fluorescein isothiocyanate-labeled anti-KJ1-26 antibodies (BD Biosciences) in 100 μl FACS buffer (PBS containing 2% bovine serum albumin and 0.02% sodium azide). The cells were washed three times in FACS buffer, and permeabilized with 100 μl Cytofix/Cytoperm (BD Biosciences) on ice for 30 min. Following three washes with Perm Wash (BD Biosciences), cells were stained with phycoerythrin-labeled anti-IL-10 antibody in 100 μl of Perm Wash on ice for 30 min. Cells were washed two times with Perm Wash and once with FACS buffer. Splenocytes from the same transgenic mice were also stained without stimulation for surface markers in FACS buffer followed by fixation with 2% paraformaldehyde. $CD4^+$ KJ1-26^+ cells were analyzed on a FACScaliber (BD Biosciences) flow cytometer using CellQuest software (BD Biosciences).

2.5. In vitro T cell proliferation

Single-cell suspensions were prepared from the spleens of C57BL/6 or DO11.10 mice and cultured in RPMI-1640 with

Hepes and L-glutamine (Cambrex, Walkersville, MD) supplemented with 10% FBS, 1% non-essential amino acids, 1% sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco Invitrogen, Carlsbad, CA) and 10 μg/ml gentamicin (Cambrex). Ninety-six-well flat-bottom plates were seeded with 4×10^5 cells per well in triplicate and stimulated with either 10 μg/ml HDM or p114–128, or 10 μg/ml OVA. Control wells contained medium alone or Con A. After 48 h of primary culture at 37 °C, ^{3}H thymidine was added to cultures for an additional 18 h. T cell proliferation was determined by measuring the incorporation of ^{3}H thymidine using a Packard Top Count (Perkin-Elmer, Downers Grove, IL) and the data were expressed as CPM of stimulated cultures.

2.6. Adoptive cell transfer

Mice were treated i.v. with three doses of p114–128 on days 0, 7 and 14. Spleens were harvested 7 days after the final immunization and single-cell splenocyte suspensions were prepared and pooled from eight mice and injected via the tail vein using a 30-gauge needle into eight recipient mice. Each animal received 5.5×10^7 cells in 0.2 ml saline at the time of adoptive transfer. Prior to adoptive transfer, recipient mice were sensitized by i.p. injection of 10 μg HDM on 1 mg of alum on days 0 and 7. Sensitized control animals received adoptive transfer of splenocytes from naïve mice. Three and five days after the adoptive transfer, recipient animals and a group of naïve mice were challenged i.t. with HDM. BALF was collected 24 h after the final challenge for differential cell counting.

2.7. Collection of BALF, differential cell count and cytokine analysis

Mice were sacrificed and the lungs were lavaged three times using 1 ml sterile saline per mouse. The BALF was centrifuged, supernatant saved for cytokine analysis and the cell pellet re-suspended in PBS containing 5% FBS. Total cells from the BALF were counted on hemacytometers by Trypan Blue exclusion, and 2.5×10^4 cells were cytospun onto microscope slides and stained with Diff-Quik (Dade Behring, Newark, DE). Cells were differentiated using light microscopy and differential cell counts were determined by multiplying the total cells per ml by the percentage of eosinophils of 400 stained and counted cells per mouse. Supernatants of BALF were assayed for Th1/Th2 cytokines using Cytometric Bead Array (CBA, BD Biosciences, San Diego, CA) according to the manufacturer's instructions.

2.8. Statistical analysis

Data from proliferation, differential cell counts and flow cytometry were analyzed using the Student *t* test. Significant differences are indicated by "*" ($P < 0.05$) or "**" ($P < 0.01$) on all figures. The data are presented as mean CPM ± S.E.M. (Fig. 1A), mean eosinophils per ml or mean total cells per ml ± S.E.M. (Figs. 1B–4A), or mean total cell number or percentage ± S.E.M. (Fig. 4).

3. Results

3.1. Administration of a single peptide to naïve mice induces peripheral tolerance and offers protection against HDM challenge

Administration of foreign antigens results in either an active immune response or immunologic tolerance. The route of administration, dose and structure of the antigen, as well as the presence of an adjuvant influence the outcome. Injection of allergen with alum stimulates Th2 responses, whereas systemic delivery of protein in the absence of an inflammatory signal is an effective way of inducing peripheral tolerance. For this reason, the i.v. route was chosen to deliver p114–128, a peptide containing a single T cell epitope of the Der p 1 protein. Peripheral tolerance was determined by measuring in vitro proliferation of splenocytes. Proliferation of splenocytes from p114–128-injected mice was minimal when stimulated with p114–128 or HDM, compared to the mice sensitized with 10 μg HDM on 1 mg alum ($P < 0.05$)

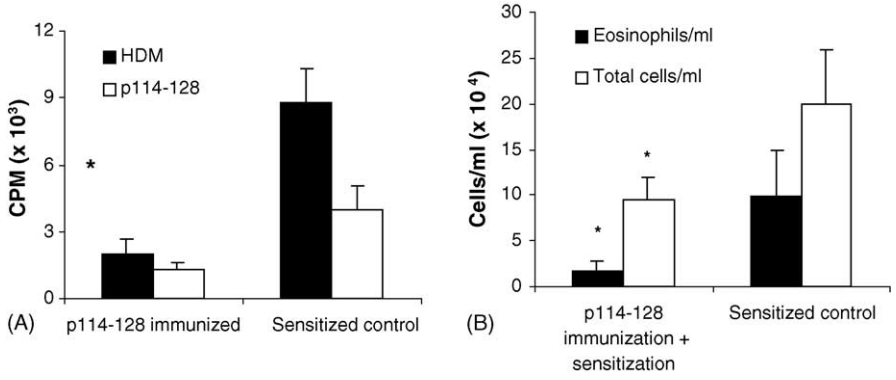

Fig. 1. Intravenous administration of peptide inhibits proliferation and protects mice against eosinophilia. (A) Proliferation of splenocytes was determined by measurement of ^{3}H thymidine uptake. (B) Differential counts of BALF cells.

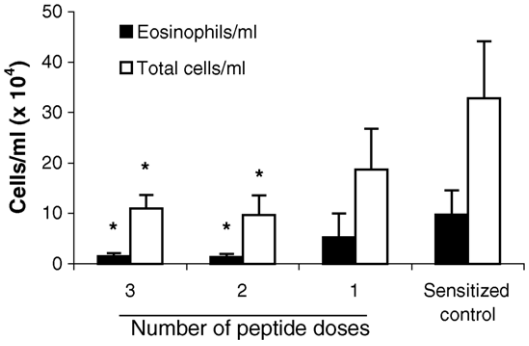

Fig. 2. Effect of peptide dose on protection in sensitized mice. Two or three doses of peptide significantly reduce the number of eosinophils and total cells in the lung.

(Fig. 1A). Minimal background proliferation was observed from stimulated naïve cells and cells stimulated with media alone (data not shown).

A challenge study was performed to determine if the peripheral tolerance induced by p114–128 would prevent the priming of Th2 cells and airway inflammation. Mice were tolerized by i.v. delivery of p114–128, sensitized by i.p. injection of 50 μg HDM on 4 mg alum and intratracheally challenged with HDM. The control group was sensitized and challenged, but did not receive peptide. There were significantly fewer eosinophils in the BALF of peptide-treated mice compared to the sensitized control ($P < 0.05$) (Fig. 1B). No eosinophils were present in the BALF of challenged naïve mice (data not shown).

Serum IgE antibody was sporadically detected in some animals in each group, including the sensitized control. The levels were too low to make a meaningful comparison between the peptide treated and the sensitized groups. The following cytokines were detected in the BALF: ??????. There were no differences in the Th1 cytokine levels between the peptide treated and sensitized animals (data not shown), suggesting that the protection was not due to a shift to Th1 response. IL-5 cytokine does not appear to correlate with the eosinophil counts.

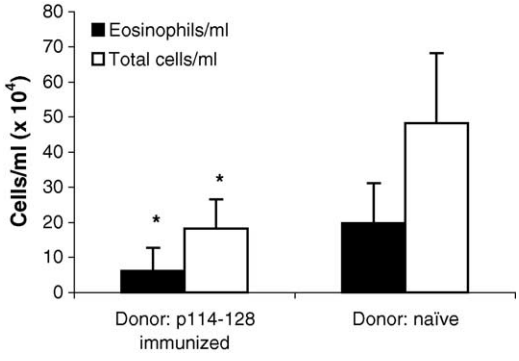

Fig. 3. Adoptive transfer of splenocytes from peptide-treated, but not naïve, mice protects recipients from increases in the number of eosinophils and total cells in the lung.

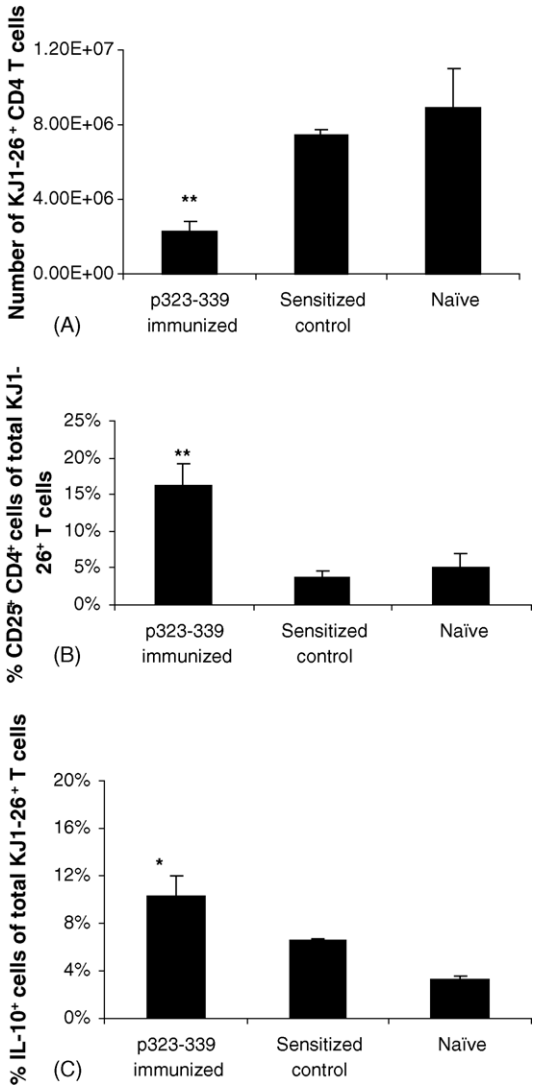

Fig. 4. Generation of $CD25^+CD4^+$ and IL-10^+ T cells following i.v. administration of OVA peptide 323–339 in DO11.10 TCR transgenic mice. Mice were treated with three i.v. doses of p323–329 or sensitized twice with OVA on alum. Spleens were harvested 1 week after the last i.v. treatment and stained directly for cell markers or stimulated before cell marker and IL-10 staining, as described in Section 2. Stained cells were subjected to flow cytometry to determine (A) total antigen-specific (KJ1-26^+) T cells, (B) percentage of antigen-specific T cells expressing CD25 and CD4 and (C) percentage of antigen-specific T cells producing IL-10.

3.2. Administration of a single peptide to sensitized mice offers protection against challenge with HDM

Allergy immunotherapy is usually prescribed for patients with an established Th2 response. We, therefore, investigated if i.v. administration of peptide could induce protection in sensitized mice. One to three doses of peptide were administered by i.v. injection to mice that had previously received sensitizations by i.p. injection of HDM on alum. Mice were then challenged by i.t. administration of HDM. Inhibition of eosinophil infiltration in the lungs was observed and the degree of protection was dependent on the number of peptide

doses. Two or three doses of p114–128 significantly reduced the eosinophil recruitment by 83.8% and 86.5%, respectively, as compared to the sensitized control ($P < 0.05$) (Fig. 2). One dose of peptide provided only partial protection (not significant). No eosinophils were detected in challenged naïve animals (data not shown).

3.3. Adoptive transfer of spleen cells from peptide-treated mice into sensitized mice induces protection against challenge with HDM

The protection conferred by peptide administration could be due to either clonal deletion or active suppression of antigen-specific T cells. To determine which mechanism was operative, the total spleen cells from peptide-treated or naïve mice were transferred to animals previously sensitized by i.p. injection of HDM adsorbed to alum. Each animal received 5.5×10^7 splenocytes via tail vein injection and 3 and 5 days later, the animals were challenged i.t. with HDM. A 70% reduction in eosinophil numbers in the BALF was observed in the mice receiving splenocytes from peptide-treated mice as compared to the sensitized control that received naïve cells ($P < 0.05$) (Fig. 3). No eosinophils were detected in challenged naïve animals (data not shown). The recipients of peptide-treated splenocytes were not directly dosed with peptide, therefore, it is likely that the protection was mediated by active suppression, rather than deletion of Th2 cells.

3.4. Peptide administration induces $CD25^+CD4^+$ and IL-10^+ T cells in OVA TCR transgenic mice

F1 mice expressing the TCR marker, KJ1-26, were generated by mating the DO11.10 KJ1-26^+ TCR transgenic mouse onto the Balb/c background. These mice were utilized to phenotype T cells following systemic exposure to peptide. Naïve mice were treated with three i.v. doses of OVA peptide 323-339, or sensitized with i.p. injection of OVA adsorbed to alum, as described in Section 2 ($n = 5$ per group). One week after the final i.v. dose, spleen and lymph nodes were harvested and directly stained for flow cytometry analysis. The total number of KJ1-26^+ $CD4^+$ T cells in the spleen (Fig. 4A) was decreased by peptide treatment compared to the sensitized controls. Despite this reduction, the number of KJ1-26^+ cells expressing CD25 and CD4 molecules (Fig. 4B) was increased after peptide treatment compared to the sensitized controls. Similar results were observed in pools of lymph node cells (data not shown). Additionally, after in vitro OVA stimulation, there was a significant increase in the percentage of IL-10-secreting antigen-specific T cell splenocytes (Fig. 4C) following peptide treatment compared to the sensitized controls. IL-10^+ and $CD25^+$ staining did not co-localize on the same cells (data not shown) indicating that i.v. peptide administration likely induces two separate T cell populations. Furthermore, T cells from peptide-treated mice were anergic when stimulated with OVA in vitro (data not shown). Taken together, these results indicate that the peptide-induced T cells may be regulatory T (Tr) cells.

4. Discussion

Allergic individuals are often allergic to multiple antigen sources and many allergen triggers are comprised of more than one antigenic component. For these reasons, it is important for allergy vaccines to be effective against complex protein sources. Induction of immunotolerance has been actively pursued for allergy immunotherapy. Tolerance to foreign antigens can be achieved through the delivery of soluble protein or peptides in the absence of inflammatory signals. A peptide-based allergy vaccine has a safety advantage over whole protein because peptide is incapable of cross-linking IgE, thus avoiding the risk of anaphylaxis.

It has been repeatedly shown, in murine models, that a MHC-binding peptide can induce immunotolerance and protection against the protein containing the peptide, a phenomenon known as linked suppression. In the HDM allergy model, inhalation of the peptide 111–139 of Der p 1 by C57BL/6 mice resulted in failure to respond to subsequent challenge [2]. Naïve mice treated with i.n. peptide and re-challenged with Der p 1/CFA s.c. secreted significantly less IL-2 than control mice when stimulated in vitro with Der p 1 or p111–139. Peptide treatment also induced tolerance in previously sensitized mice. Tolerant mice re-challenged with i.n. p111–139 showed a transient $CD4^+$ T cell activation followed by a down-regulation of IL-2, IL-3/GM-CSF and IFN-γ responses after in vitro stimulation with p111–139 [3]. Lymph node cells had reduced ability to respond to Der p 1 and OVA in vitro after i.n. peptide administration followed by immunization with Der p 1 in CFA plus OVA [4]. Despite these data, however, it remained unclear if tolerance induced by a single peptide would be sufficient to induce in vivo protection against complex allergens containing multiple proteins.

In the present studies, intravenous administration of a single MHC-binding peptide was sufficient to prevent eosinophil-mediated airway inflammation and intervene in an established allergic state in sensitized animals. The sensitization and airway challenge used crude HDM extract that contains several proteins including Der p 1, Der p 2 and others, whereas the Der p 1 peptide 114–128 was used in immunizations. This peptide represents a minimal epitope [4], which may overlap with the major epitope in the HDM extract. Taken together, these data and the nature of the peptide used suggest that peptide-based allergy vaccines may not need to contain all T cell epitopes.

Immunotolerance may be orchestrated by clonal deletion or anergy of allergen-reactive T cells or by active suppression mediated by regulatory T cells. Several types of Tr cells have been identified, including $CD4^+CD25^+$ populations and $CD4^+$ subsets secreting IL-10 (Tr1) or TGF-β (Th3). $CD4^+CD25^+$ T cells have been implicated in the protection of mice from colitis through their immunoregulatory functions

[9,10]. Moreover, a $CD4^+CD25^+$ T cell subset with regulatory properties has been identified in humans [11]. Generation of anergic $CD4^+$ $CD45RB^{low}$ T cells with suppressive properties was found to be dependent on the presence of IL-10. This T cell population inhibited the development of colitis and produced high levels of IL-10 [12,13]. IL-10 production and tolerance induction have also been linked to peptide immunotherapy. Sensitized mice were protected from airway hyper-reactivities following an inhalant Der p 1 allergen exposure when immunized i.n. with Der p 1 peptide 111–139 [5]. Additionally, $CD4^+$ T cells exhibited a tolerized phenotype with increased IL-10 production and a concomitant decrease in IL-4, IL-5 and IL-13. Generation of IL-10-secreting, anergic T cells following successful cat [6] or bee/wasp [14] peptide immunotherapy has been reported in human clinical trials.

Tr cells are induced in an antigen-specific manner, but can exert suppressive effects non-specifically, a process termed bystander suppression. In the present study using the mouse HDM model, adoptive transfer of spleen cells from peptide-treated mice conferred protection to sensitized animals, whereas sensitized recipients of naïve splenocytes developed a profound eosinophilia after challenge. Therefore, we hypothesize that protection against whole allergen is mediated by Tr cells induced by peptide immunization. To demonstrate this, the frequency of $CD4^+CD25^+$ spleen cells in the peptide immunized and the sensitized mice were determined to be 3.2% and 2.8%, respectively. The low frequency of antigen-specific T cells in the C57BL/6 mice made it impossible to isolate and characterize the function of $CD4^+CD25^+$ T cells following peptide treatment. Therefore, the relationship between i.v. peptide administration and the generation of Tr cells was investigated using the DO11.10 mouse strain that is transgenic for the OVA TCR ($KJ1\text{-}26^+$). Peptide administration in this model was associated with an increase in two distinct antigen-specific populations that were either $CD25^+CD4^+$ or $IL\text{-}10^+$. These results indicate that IL-10-secreting T cells and $CD4^+CD25^+$ T cells may operate in concert to maintain tolerance induced by i.v. peptide. Two similar Tr subsets have previously been shown to co-exist in a mouse model of inflammatory bowel disease [15].

The role these cell populations play in the mechanism of protection observed in these studies needs to be established. An important issue is to determine the function of these two separate classes of T cells and demonstrate their ability to inhibit T cell stimulation in vitro. Additionally, further characterization of the T cells is required to distinguish them from effector cells. These studies may reveal that intravenous peptide injection induces Tr cells that function through bystander suppression and thus can provide protection against a multi-component allergen trigger. The finding that a single peptide can induce protection to a whole allergen molecule in inbred mouse models suggests that it is not necessary for a peptide-based allergy vaccine to include all immunogenic epitopes. However, designing a peptide vaccine for human is complicated by the polymorphism of the human MHC molecules. In order for a peptide vaccine to provide protection for a majority of humans, a peptide cocktail would still be needed.

References

[1] Robinson DS, Hamid Q, Ying S, et al. Predominant Th2-like bronchoalveolar T-lymphocyte population in atopic asthma. N Engl J Med 1992;326(5):298–304.

[2] Hoyne GF, O'Hehir RE, Wraith DC, Thomas WR, Lamb JR. Inhibition of T cell and antibody responses to house dust mite allergen by inhalation of the dominant T cell epitope in naive and sensitized mice. J Exp Med 1993;178(5):1783–8.

[3] Hoyne GF, Askonas BA, Hetzel C, Thomas WR, Lamb JR. Regulation of house dust mite responses by intranasally administered peptide: transient activation of $CD4^+$ T cells precedes the development of tolerance in vivo. Int Immunol 1996;8(3):335–42.

[4] Jarnicki AG, Tsuji T, Thomas WR. Inhibition of mucosal and systemic Th2-type immune responses by intranasal peptides containing a dominant T cell epitope of the allergen Der p 1. Int Immunol 2001;13(10):1223–31.

[5] Hall G, Houghton CG, Rahbek JU, Lamb JR, Jarman ER. Suppression of allergen reactive Th2 mediated responses and pulmonary eosinophilia by intranasal administration of an immunodominant peptide is linked to IL-10 production. Vaccine 2003;21(5–6):549–61.

[6] Oldfield WL, Larche M, Kay AB. Effect of T-cell peptides derived from Fel d 1 on allergic reactions and cytokine production in patients sensitive to cats: a randomised controlled trial. Lancet 2002;360(9326):47–53.

[7] Oldfield WL, Kay AB, Larche M. Allergen-derived T cell peptide-induced late asthmatic reactions precede the induction of antigen-specific hyporesponsiveness in atopic allergic asthmatic subjects. J Immunol 2001;167(3):1734–9.

[8] Muller U, Akdis CA, Fricker M, Akdis M, Blesken T, Bettens F, et al. Successful immunotherapy with T-cell epitope peptides of bee venom phospholipase A2 induces specific T-cell anergy in patients allergic to bee venom. J Allergy Clin Immunol 1998;101(6 Pt 1):747–54.

[9] Annacker O, Pimenta-Araujo R, Burlen-Defranoux O, Barbosa TC, Cumano A, Bandeira A. $CD25^+CD4^+$ T cells regulate the expansion of peripheral CD4 T cells through the production of IL-10. J Immunol 2001;166(5):3008–18.

[10] Read S, Malmstrom V, Powrie F. CytotoxicT lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. J Exp Med 2000;192(2):295–302.

[11] Jonuleit H, Schmitt E, Stassen M, Tuettenberg A, Knop J, Enk AH. Identification and functional characterization of human CD4(+)CD25 (+) T cells with regulatory properties isolated from peripheral blood. J Exp Med 2001;193(11):1285–94.

[12] Asseman C, Mauze S, Leach MW, Coffman RL, Powrie F. An essential role for interleukin-10 in the function of regulatory T cells that inhibit intestinal inflammation. J Exp Med 1999;190(7):995–1004.

[13] Groux H, O'Garra A, Bigler M, et al. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nature 1997;389(6652):737–42.

[14] Akdis CA, Blesken T, Akdis M, Wuthrich B, Blaser K. Role of interleukin-10 in specific immunotherapy. J Clin Invest 1998;102(1):98–106.

[15] Foussat A, Cottrez F, Brun V, Fournier N, Breittmayer JP, Groux H. A comparative study between T regulatory type 1 and $CD4^+CD25^+$ T cells in the control of inflammation. J Immunol 2003;171(10):5018–26.