Amelioration of ovalbumin-induced allergic airway disease following Der p 1 peptide immunotherapy is not associated with induction of IL-35

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In the present study, we show therapeutic amelioration of established ovalbumin (OVA)-induced allergic airway disease following house dust mite (HDM) peptide therapy. Mice were sensitized and challenged with OVA and HDM protein extract (*Dermatophagoides* species) to induce dual allergen sensitization and allergic airway disease. Treatment of allergic mice with peptides derived from the major allergen Der p 1 suppressed OVA-induced airway hyperresponsiveness, tissue eosinophilia, and goblet cell hyperplasia upon rechallenge with allergen. Peptide treatment also suppressed OVA-specific T-cell proliferation. Resolution of airway pathophysiology was associated with a reduction in recruitment, proliferation, and effector function of T_H2 cells and decreased interleukin (IL)-17⁺ T cells. Furthermore, peptide immunotherapy induced the regulatory cytokine IL-10 and increased the proportion of Fox p3⁺ cells among those expressing IL-10. Tolerance to OVA was not associated with increased IL-35. In conclusion, our results provide *in vivo* evidence for the creation of a tolerogenic environment following HDM peptide immunotherapy, leading to the therapeutic amelioration of established OVA-induced allergic airway disease.

INTRODUCTION

Allergen-specific immunotherapy, which targets the underlying mechanisms of allergic inflammation, is disease-modifying and remains the only curative approach for allergic diseases.^{1,2} However, administration of whole-allergen molecules in current specific immunotherapy products carries the risk of IgE-mediated adverse events, which may be local or systemic and may include life-threatening anaphylaxis.^{3,4} Many novel approaches are being designed to reduce the allergenicity of immunotherapy preparations while maintaining immunogenicity. One approach is the use of short synthetic peptides that represent dominant T-cell epitopes of the allergen. Short peptides exhibit markedly reduced capacity to cross-link IgE and activate mast cells and basophils, owing to a lack of tertiary structure. Studies in mouse models have established the feasibility of this approach, and clinical studies are currently in progress in both allergic and autoimmune

diseases.^{5,6} Peptide immunotherapy induces immunological tolerance mediated by regulatory T cells and interleukin (IL)-10, which suppresses allergen-specific T-cell proliferation and production of T_H2 cytokines.^{5,7–10}

Allergen proteins contain multiple T-cell epitopes restricted by a wide range of human leukocyte antigen (HLA) molecules. Furthermore, many allergen sources contain multiple allergenic proteins. For peptide immunotherapy products, the diversity present in both allergen molecules and HLA molecules requires that multiple immunodominant T-cell epitopes be represented to achieve population coverage. Peptide solubility and, in some cases, regulatory requirements (such as the ability to resolve individual peptide components from a mixture) constrain the numbers of individual peptides that can be formulated for treatment. Therefore, ideally, an effective peptide therapy should be capable of generating a local environment (e.g., through the induction of regulatory T cells)

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Received 12 September 2012; accepted 1 July 2013; published online 14 August 2013. doi:10.1038/mi.2013.56

that can facilitate the induction of tolerance to multiple allergen proteins encountered contemporaneously. The demonstration that such a “tolerogenic environment” can be achieved *in vivo* within the respiratory mucosa will inform development of future interventions in allergic diseases.

In a recent study in the context of allergic asthma, we provided evidence of an association of peptide immunotherapy-induced immunological tolerance with linked epitope suppression.⁵ In this case, tolerance was induced via intradermal, rather than airway, delivery of peptides. Others have shown that tolerance to an allergen (induced via the airways) can prevent sensitization (i.e., prophylaxis) to an unrelated antigen.¹¹ However, to be effective in the field, such an approach must be applicable to existing allergic airway sensitizations (i.e., therapeutic). This issue has yet to be addressed in the literature.

In the current study, we hypothesized that mice co-sensitized to house dust mite (HDM) and ovalbumin (OVA), treated by mucosal delivery of T-cell epitopes from the HDM allergen Der p 1 to create a tolerogenic environment and then concomitantly exposed (via the airways) to both allergens, would exhibit reduced allergic airway responses to OVA upon inhaled OVA challenge.

We provide evidence that peptide immunotherapy reduced airway hyperresponsiveness (AHR), OVA-specific T-cell proliferation, T_H2-cell numbers, and pro-inflammatory cytokines. Modulation of the response to OVA was associated with induction of IL-10 production and Fox p3 expression, but not increased IL-35 production.

RESULTS

Der p 1 peptide immunotherapy improves lung function and reduces inflammation in the lungs of OVA-exposed, dual allergen-sensitized mice

To investigate whether peptide immunotherapy decreased airway inflammation, mice were killed and airway inflammation was investigated in lung tissue and in the airway (bronchoalveolar lavage (BAL) cell counts). Hematoxylin and eosin and periodic acid-Schiff stainings of fixed lung tissue sections were performed to identify eosinophils and mucous-secreting goblet cells. Histological observations of lung tissue showed that mice that were sham-sensitized, but challenged with allergen, did not exhibit any pulmonary inflammation (Figure 1a,d). Animals sensitized and treated with vehicle demonstrated extensive peribronchial eosinophilic inflammatory infiltrates (Figure 1b) with airway luminal hyperplasia of goblet cells (Figure 1e). Peptide immunotherapy significantly reduced both eosinophilia (Figure 1c) and goblet cell hyperplasia (Figure 1f).

Total lymphomononuclear cell numbers in lung tissue digests were modestly, but significantly, decreased with peptide immunotherapy (Figure 1g). OVA airway challenge was associated with a significant increase in peribronchial eosinophil numbers from vehicle-treated mice. Treatment with Der p 1 peptides significantly reduced eosinophilia (Figure 1h). Similarly, peptide treatment significantly

reduced the number of goblet cells (Figure 1i). Total and differential inflammatory BAL-fluid cell counts were performed by Wright–Giemsa staining of cytospin slides. In the airways of vehicle-treated mice, total BAL inflammatory cells were increased compared with unsensitized mice (Figure 1j). Of these cells, eosinophils constituted $56.08 \pm 11.8\%$, with the remaining cells being neutrophils ($7.1 \pm 2.5\%$), lymphocytes ($5.9 \pm 1.1\%$), and macrophages ($41.3 \pm 9.3\%$). Peptide immunotherapy had no significant effect on BAL cell counts (total or differential).

To investigate whether peptide immunotherapy improved lung function in dual allergen- (HDM/OVA) sensitized mice, we measured airway responsiveness to MCh challenges using the *FlexiVent* system (Scireq, Montreal, QC, Canada) 48 h after the last OVA rechallenge. Allergen-sensitized mice treated with vehicle demonstrated MCh dose-dependent increases in total respiratory system resistance (R_{RS}), which were significantly reduced in mice treated with the Der p 1 peptides (Figure 1k, lung resistance; Figure 1l, area under the curve).

Der p 1 peptide immunotherapy reduces lymphocyte IL-5 production and T_H2-lymphocyte recruitment to the lungs of OVA-exposed, dual allergen-sensitized mice

Although (like eosinophils) airway luminal lymphocyte numbers were not modified by peptide immunotherapy (Figure 1j), total CD4⁺ cell numbers in lung tissue digests were significantly reduced compared with vehicle-treated mice (Figure 2a). The numbers of tissue CD8⁺ cells and tissue B cells (CD19⁺) were also significantly reduced with peptide treatment compared with vehicle treatment (Figure 2b,c). Given the pivotal role T_H2 cells are believed to have in allergic disease, we performed flow-cytometry analysis of lung tissue digest cells to quantify T_H2 cells along with inflammatory cytokine-producing cells. CD4⁺ cells were gated and further analyzed for T1/ST2 expression to identify T_H2 cells (T1/ST2⁺ CD4⁺). Total T1/ST2⁺ CD4⁺ cell numbers were significantly reduced in peptide-treated, compared with vehicle-treated, dual allergen-sensitized mice (Figure 2d,f). Furthermore, IL-5-producing T_H2 cells (IL-5⁺ T1/ST2⁺ CD4⁺) were decreased by approximately 50% (Figure 2e,f). No decrease in both percent and total IL-4⁺ CD4⁺ cell numbers were observed in this study after peptide treatment. IL-13 levels were not measured.

Der p 1 peptide immunotherapy decreases IL-17⁺ cell recruitment to the lungs of OVA-exposed, dual allergen-sensitized mice

Previous studies have implicated the pro-inflammatory cytokine IL-17 in the development of airway inflammation and AHR. Therefore, we investigated intracellular expression of IL-17 in this model by flow cytometry and assessed the influence of peptide treatment. Peptide immunotherapy reduced the number of IL-17⁺ cells in lung tissue digests (Figure 3a). To examine IL-17-producing CD4⁺ cells, gated CD4⁺ cells were analyzed for IL-17 expression. Both the percentage of IL-17⁺ CD4⁺ cells and the total number of IL-17⁺ CD4⁺ cells in lung tissue digests were decreased in peptide-treated mice,

Figure 1 Der p 1 peptide immunotherapy reduces cell recruitment to the lung and airway hyperresponsiveness in ovalbumin-challenged, dual allergen-sensitized mice. Mice were sensitized and challenged as described in Methods. (a–f) Representative lung histology from control, vehicle-, and peptide-treated mice showing inflammatory infiltrates and goblet cells. Lung sections were stained with hematoxylin and eosin (H&E; a–c) or periodic acid-Schiff (PAS; d–f). (g) Single lobes of lung tissue were enzymatically digested to generate cell suspensions for the enumeration of total numbers of cells in lung tissue digest (g). Tissue eosinophils (h) and mucin⁺ cells (i) in the submucosa were enumerated using a standardized protocol. (j) Total and differential cell counts were performed on bronchoalveolar lavage (BAL) fluids using Wright–Giemsa-stained cytospin preparations to obtain absolute numbers of eosinophils (Eos), neutrophils (Neu), lymphocytes (Lymph), and macrophages (Mac). Percentages were multiplied by the total number of cells obtained in the lavage fluid. On day 58, airway responsiveness was measured using the *FlexiVent* small animal ventilator system. (k) Total respiratory system resistance (cm $\text{H}_2\text{O/ml/s}$) in response to increasing doses of nebulized methacholine was assessed ($n=8$). (l) The area under the methacholine dose–response curve (AUC) is shown. * $P<0.05$ vs. vehicle-treated mice.

compared with vehicle-treated mice (Figure 3b,c). Peptide treatment also reduced total IL-17⁺ CD4⁺ cell numbers ($3.27 \pm 0.49 \times 10^4$ vs. $2.04 \pm 0.23 \times 10^4$, $P<0.05$) in draining lymph node (DLN) compared with vehicle treatment.

Der p 1 peptide immunotherapy reduces OVA-specific T-cell proliferation

To assess the effect of Der p 1 peptide immunotherapy on functional T-cell responses to allergen, OVA- and HDM-

specific T-cell proliferation was measured using two independent methods: (a) [³H] thymidine assay and (b) flow cytometry following labeling with the cell division-tracking dye carboxy-fluorescein diacetate succinimidyl ester (CFSE). DLN cells isolated from vehicle-treated and peptide-treated mice were cultured with different concentrations of OVA and HDM. In both sets of experiments, Der p 1 peptide treatment significantly reduced OVA-induced proliferation (Figure 4a,b). HDM induced less proliferation than OVA, and although

Figure 2 Der p 1 peptide immunotherapy reduces recruitment of lymphoid effector cells to the lung following OVA challenge. Mice were killed 48 h after ovalbumin rechallenge (i.e., day 58), and cells were isolated from lung tissue digest as described in Methods. Cells were stained for surface markers and intracellular cytokines. For intracellular interleukin (IL)-5 staining, cells were stimulated with phorbol 12-myristate 13-acetate and ionomycin in the presence of brefeldin A for 6 h. **(a)** Total CD4⁺ cells. **(b)** Percentage of T1ST2⁺ CD4⁺ cells (left panels) and percentage of IL-5⁺ cells in T1ST2⁺ CD4⁺ cells (center panels), total T1ST2⁺ CD4⁺ cells (top right panel), and total IL-5⁺ T1ST2⁺ CD4⁺ (bottom right panel) are shown. **(c)** Total IL-4⁺ CD4⁺ cells, **(d)** total CD8⁺ cells, and **(e)** total CD19⁺ cells are shown. **(f)** Flow-cytometry analysis showing percent T1ST2⁺ CD4⁺ cells (left panels) and percent IL-5⁺ cells in T1ST2⁺ CD4⁺ cells (right panels). T1ST2⁺ CD4⁺ cells shown in left panels are after gating CD4⁺ cells. Data are expressed as mean cell numbers $\pm$ s.e.m. of each population by multiplying the percentage expression on total live cells acquired by the total cell counts. Cells of lung tissue digest from 2–3 mice were pooled; each bar shows the mean $\pm$ s.e.m. of 3–4 data points from eight mice. ND: intracellular cytokines in saline group were not quantified due to insufficient cell numbers in lung tissue digests. * $P < 0.05$ vs. vehicle-treated mice.

peptide treatment was associated with smaller proliferative responses, the difference between peptide and vehicle was not significant using either method. Representative data plots of CFSE-labeled DLN cell cultures stained for CD4 demonstrate that both OVA and HDM induced substantial proliferation of CD4 cells isolated from vehicle-treated mice (**Figure 4b**). Peptide immunotherapy markedly reduced OVA-driven CD4 cell proliferation, while also resulting in a more modest reduction in HDM-driven CD4 cell proliferation.

Der p 1 peptide immunotherapy increases IL-10, but not IL-35, in the lungs of OVA-exposed dual allergen-sensitized mice

IL-10 and IL-35 protein levels were examined in BAL fluids and supernatants of lung tissue digests (and serum; IL-35 only) using enzyme-linked immunosorbent assay (ELISA). Peptide immunotherapy was associated with a significant, although modest, increase in IL-10 protein in supernatants of lung tissue digest (SLD; **Figure 5a**). The concentration of IL-10 was significantly

lower in BAL fluids than in lung tissue digest and did not change after peptide immunotherapy (**Figure 5a**). As IL-10 can be produced by many cell types, we performed intracellular IL-10 analysis in lung tissue digest cells to investigate which lung cells produced IL-10. Peptide immunotherapy was associated with a significant increase in both percentage (**Figure 5b**, representative plots) and total (**Figure 5c**) IL-10⁺ lung tissue digest cells. To determine which cells produced IL-10, positive cells were gated and further analyzed for expression of CD4 (T-helper cells) and CD19 (B cells). In vehicle-treated mice, CD4⁺ and CD19⁺ cells constituted $26 \pm 2.6\%$ and $39 \pm 6.5\%$ of IL-10⁺ cell population, respectively. In peptide-treated mice, the percentage of IL-10⁺CD4⁺ cells, but not IL-10⁺CD19⁺ cells, increased significantly following peptide immunotherapy (**Figure 5d**). IL-10⁺ cells also increased significantly in the DLN of peptide-treated mice ($3.4 \pm 0.84\%$ vs. $1.3 \pm 0.15\%$, $P < 0.05$). In DLN of vehicle-treated mice, CD4⁺ and CD19⁺ cells constituted $52.6 \pm 2.7\%$ and $27.9 \pm 3.4\%$, respectively, of the IL-10⁺ cell population. Peptide treatment also significantly increased the

Figure 3 Der p 1 peptide immunotherapy decreases interleukin (IL)-17⁺ cells in lung following ovalbumin challenge. Mice were killed 48 h after OVA rechallenge. Cells isolated from lung tissue digest as described in Methods were cultured with phorbol 12-myristate 13-acetate and ionomycin in the presence of brefeldin A for 6 h. (a) Representative examples of IL-17 staining in lung digest lymphocytes. (b) CD4 gating (left panels) and percentage IL-17⁺ cells within the CD4⁺ population (right panel). (c) Total numbers of IL-17⁺ CD4⁺ cells. Data are expressed as mean $\pm$ s.e.m. by multiplying the percentage expression on CD4⁺ cells in total live cells acquired by the total CD4⁺ cell counts. * P < 0.05 vs. vehicle-treated mice.

CD4⁺ population within IL-10⁺ cells ($63.2 \pm 4.1\%$, P < 0.05, vs. vehicle-treated mice) without any significant change in CD19⁺ cell population ($30.9 \pm 1.4\%$). Peptide immunotherapy was not associated with an increase in IL-35 in BAL, lung tissue digest, or serum (Figure 5e).

Increased expression of Fox p3 after Der p 1 peptide immunotherapy

Peptide therapy increased IL-10 expression in CD4⁺ cells in lung tissue digest (Figure 6a). As expression of IL-10 can be associated with Fox p3⁺ CD4⁺ Treg cells and their function, we further investigated the Fox p3⁺ subpopulation within IL-10⁺ CD4⁺ cells. IL-10⁺ CD4⁺ lung digest cells were gated and analyzed for Fox p3 expression. A significantly greater proportion of IL-10⁺ CD4⁺ cells were Fox p3⁺ in peptide-treated mice, compared with vehicle-treated mice (Figure 6a,b). A similar effect was seen in the DLN where a significantly lower percentage of Fox p3⁺ CD4⁺ cells ($12.4 \pm 1.8\%$) was present in the IL-10⁺ population from vehicle-treated mice compared with peptide-treated mice ($28.45 \pm 6.67\%$, P < 0.05). Fox p3 has been described as a marker of regulatory T cells, and

approximately 15–20% of the CD4⁺ cells in lung tissue digest and DLN of vehicle-treated mice expressed Fox p3. Interestingly, examination of the CD4⁺ population irrespective of IL-10 expression yielded no difference in the percentage of Fox p3⁺ between the treatment groups (Figure 6c). As determined by quantification of mean fluorescence intensity, the intensity of Fox p3 expression within lung digest CD4⁺ cells was significantly increased in lung tissue digest cells from peptide-treated mice compared with vehicle-treated mice, indicating that individual cells expressed more Fox p3 following peptide immunotherapy (Figure 6d). Similarly, peptide treatment enhanced mean fluorescence intensity of Fox p3 in DLN (485.5 ± 19.6 vs. 804.7 ± 63.2 in vehicle- and peptide-treated mice, respectively, P < 0.05).

DISCUSSION

The ability to confer tolerance to a protein through creation of a local tolerogenic environment, after treating with one or more epitopes from another protein, has significant clinical potential, particularly in allergic and autoimmune diseases. To be effective in clinical practice, this approach must work in

Figure 4 Der p 1 peptide immunotherapy decreases ovalbumin (OVA)-stimulated proliferation of draining lymph node (DLN) cells isolated from dual allergen-exposed mice. Mice were killed 48 h after OVA rechallenge. *In vitro* proliferation assays of DLN cells were performed by [^3H] thymidine incorporation and carboxyfluorescein diacetate succinimidyl ester (CFSE). Cells were cultured in the presence of OVA, house dust mite (HDM), or medium alone for 6 days. **(a)** Proliferation of DLN cells was cultured with OVA or HDM extract, as correlated with [^3H] thymidine incorporation; data are expressed as mean stimulation index $\pm$ s.e.m. ($n=8$). **(b)** Proliferation of DLN cells was cultured with OVA or HDM extract, as correlated with CFSE dilution in CD4⁺ cells. Plots show the proliferation of CD4⁺ cells. Numbers in the plots indicate percentage of CD4⁺ cells proliferated following treatment. Representative plots are from one of the eight mice with similar results. * $P<0.05$ vs. vehicle-treated mice.

established disease. However, to date, published studies have only demonstrated prevention of *de novo* sensitization to unrelated antigens. For example, Van Hove *et al.*¹¹ induced enduring tolerance to OVA by chronic administration (8 weeks by aerosol). Re-immunization to OVA (with adjuvant) after this time was unable to elicit a recall response to OVA. Furthermore, immunization with an unrelated antigen (hen egg lysozyme) after the induction of tolerance with OVA was unable to elicit a primary T_H2 response to hen egg lysozyme. Thus, the induction of prophylactic tolerance (through the airways) with one protein can prevent subsequent T_H2 sensitization to another.

In a murine model of established, OVA-induced allergic airway disease, we show that peptide immunotherapy, with T-cell epitopes from the unrelated HDM allergen Der p 1, can reduce T_H2 inflammation and the consequences of such inflammation *in vivo*. Peptide immunotherapy reduced AHR, total lung cell numbers, tissue eosinophilia, goblet cell hyperplasia, T_H2 cells, pro-inflammatory cytokines IL-5 and IL-17, and OVA-specific T-cell proliferation. These decreases

in inflammatory outcomes were associated with increased Fox p3 expression and increased IL-10 production by T cells, but not with increased levels of IL-35, suggesting that IL-35-independent mechanisms of mucosal tolerance exist. Der p 1 peptide immunotherapy also reduced recruitment of B cells to the lung following OVA challenge. Although modulation of B-cell antibody production has been reported in some models of peptide immunotherapy,^{5,9,12,13} this is the first demonstration of modulation of B-cell recruitment to the airways.

Peptide immunotherapy with CD4 T-cell epitopes has been extensively evaluated in mouse models to prevent and treat a variety of antigen-specific inflammatory responses,^{14–20} and several clinical trials have been, or are currently being, performed in allergy and autoimmunity.^{21–36} Ideally, effective peptide therapies should generate a limited local network of tolerance that would allow expansion of T-cell tolerance beyond the treatment epitopes to other antigens encountered contemporaneously. Such a phenomenon is hypothesized to occur through the dominant action of regulatory T cells on effector T cells encountering cognate ligand at the surface of the

Figure 5 Der p 1 peptide immunotherapy increases interleukin (IL)-10 production in lung. Mice were killed 48 h after ovalbumin rechallenge, and lung tissue was digested as described in Methods. IL-10 was quantified in supernatants of lung tissue digest (SLD) and bronchoalveolar lavage fluid (BALF) by enzyme-linked immunosorbent assay. Intracellular expression of IL-10 in lung digest cells was evaluated by flow cytometry. (a) IL-10 protein levels in SLD and BALF. Data are expressed as mean $\pm$ s.e.m. ($n=8$). (b) Intracellular IL-10 expression in CD4⁺ T cells and CD19⁺ B cells. Cells were cultured with phorbol 12-myristate 13-acetate and ionomycin in the presence of brefeldin A for 6 h. Left panels show percent IL-10⁺ cells and right panels show percentage of IL-10 contributed by CD4⁺ and CD19⁺ cells. Numbers in each quadrant indicate percent cells in that quadrant. (c) Total IL-10⁺ cells in lung tissue digest. Data are expressed as mean total IL-10⁺ cell numbers $\pm$ s.e.m. by multiplying the percentage expression on total live cells acquired by the total cell counts. (d) Percentage of CD4⁺ and CD19⁺ cells in IL-10⁺ population. Cells of lung tissue digest from 2–3 mice were pooled. Each bar shows the mean $\pm$ s.e.m. of 3–4 data points from eight mice. (e) IL-35 protein levels in the BAL, supernatants of lung tissue digest and serum. Data are expressed as mean $\pm$ s.e.m. ($n=8$). * $P<0.05$ vs. vehicle-treated mice.

same antigen-presenting cell (APC).^{37,38} Although expansion of tolerance to include antigens from pathogens present in the local environment at the time of treatment is a potential outcome of this phenomenon, our clinical studies to date indicate that systemic recall responses (to purified protein derivative from *Mycobacterium tuberculosis* in an European population and *Candida albicans* extract in a North American population) are not compromised through peptide therapy (ref. 5 and unpublished data).

In the present study, mice were sensitized with both OVA and HDM extract and challenged intranasally (i.n.) with OVA and HDM to create established allergic airway disease driven independently by both allergens. Sensitization with OVA generated OVA-specific cells and i.n. challenged OVA-induced localization of OVA-specific T cells to the lung. Similarly, HDM-specific cells were also generated and localized to the lung through repeated intranasal challenge. Conceptually, inhaled challenge with OVA immediately after treatment with inhaled Der p 1 peptides resulted in presentation of T-cell epitopes from both proteins on the same local APCs, leading to

the recruitment of both tolerant HDM-specific T cells and OVA-specific effector cells, the latter coming under the influence of the former resulting in suppression of responses to OVA.

Peptide treatment reduced AHR, a cardinal feature of asthma. It also reduced total cell numbers, goblet cell hyperplasia, and eosinophilic inflammatory infiltrates in lung tissue. Despite the reduction in inflammatory cells in lung tissue, no change in cellularity was observed in the airway lumen. This dissociation between airway inflammatory cells and parameters of lung function has been observed previously. For example, in patients with allergic asthma, the number of inflammatory cells in BAL did not correlate with the degree of AHR.^{39,40} Furthermore, the failure of an anti-IL-5 intervention to improve clinical outcomes in human asthmatic subjects was associated with a failure to fully deplete parenchymal, rather than BAL, eosinophils.⁴¹ In contrast, Lemiere *et al.*⁴² demonstrated that high sputum eosinophil counts, rather than numbers of eosinophils in bronchial biopsies, were associated with higher rates of disease exacerbation.

Figure 6 Der p 1 peptide immunotherapy induces interleukin (IL)-10 expression in Fox p3⁺ CD4⁺ cells in the lung following ovalbumin (OVA) rechallenge. Mice were killed 48 h after OVA rechallenge, and cells were isolated from lung tissue digest as described in Methods. Cells were stimulated with phorbol 12-myristate 13-acetate and ionomycin in the presence of brefeldin A for 6 h. **(a)** Representative plots of flow-cytometry analysis showing expression of Fox p3 in IL-10⁺ CD4⁺ cells. Gated CD4⁺ cells were analyzed for IL-10⁺ cells (left panels) that were further analyzed for Fox p3 expression (right panels). Numbers adjacent to outlined areas indicate percent cells in each gate. **(b)** Fox p3⁺ cell population in IL-10⁺ CD4⁺ cells in lung tissue digest. **(c)** Fox p3⁺ CD4⁺ cells in lung tissue digest. **(d)** Mean fluorescence intensity of Fox p3 expression in CD4 cells in lung tissue digest. Each bar in panels **b–d** shows mean $\pm$ s.e.m. ($n=8$). * $P<0.05$ vs. vehicle-treated mice.

OVA has been widely used in murine models of allergic airway disease, inducing AHR, T_H2 responses, and inflammatory airway infiltrates.⁴³ Earlier studies in a dual HDM/OVA sensitization model demonstrated that responses to OVA in such a system are not dependent on responses to HDM and that the two responses are independent and additive in nature.⁴⁴ Thus, merely reducing responses to HDM would not account for reductions in the responses to OVA. Reduction in T_H2-cell responses and AHR in the present dual allergen-sensitization model shows that immunotherapy with HDM peptides can, under the appropriate conditions, suppress established OVA-induced allergic responses. These results are indicative of active regulation.

Peptide therapy with HDM peptides reduced T_H2-cell (T1/ST2⁺ CD4⁺) recruitment after OVA airway challenge. These cells are thought to contribute to AHR by release of pro-inflammatory cytokines and are critical effectors of allergic responses.^{45,46} A recent study also suggested that the T1/ST2⁺ pathway, rather than the eosinophil, is involved in the induction of AHR,⁴⁷ supporting earlier studies by Foster and colleagues.⁴⁸ Therefore, modulation of T_H2-cell function could be of therapeutic benefit in allergic disease. We are currently investigating how peptide immunotherapy modulates allergen-specific

T-cell function by using HLA-DR4 tetramers in two experimental models of allergic disease performed in HLA-DR4 transgenic mice. These studies will allow us to further define frequency, phenotype, gene expression, and epigenomic modification of the cells targeted during therapy.

Peptide treatment also reduced IL-5-producing T_H2 cells in the lung. However, we could not detect any difference in the numbers of IL-4-producing CD4⁺ cells in the lung between peptide-treated and vehicle-treated mice. We did not measure IL-13 in this study. A previous study showed that treatment with anti-IL-4 and IL-13 antibody did not completely abrogate AHR, T_H2 cells, and mucous production in OVA-sensitized-challenge mice.⁴⁷ Thus, murine mechanisms of AHR independent of IL-4 and IL-13 exist (e.g., IL-5-dependent accumulation of eosinophils in the lung), which can be modulated by peptide therapy.

IL-17, another pro-inflammatory cytokine produced by CD4⁺ effector cells, is thought to potentiate allergic inflammation. IL-17-producing cells (T_H17) are increased in patients with allergic asthma.⁴⁹ The absence of AHR in airway sensitized IL-17ra^{-/-} mice, and induction of AHR via airway delivery of exogenous IL-17 has demonstrated that IL-17 is required for AHR.⁵⁰ In the present study, we also detected IL-17-producing

cells in lung tissues of dual allergen-sensitized mice, and these were significantly decreased with peptide immunotherapy.

Peptide immunotherapy reduced not only T_H2 cells but also $CD8^+$ T and B cells. Previous studies suggest that not only $CD4^+$ cells but also $CD8^+$ cells, particularly a subset of $CD8^+$ cells that produce IL-4, IL-5, and IL-13 (but not interferon- γ), may be essential for the development of AHR and allergic inflammation.^{51–53} A recent study demonstrated increased numbers of $CD8^+$ cells in the lung tissue of asthmatics.⁵⁴ Thus, reduction in $CD8^+$ cells via peptide immunotherapy may contribute to efficacy and should be evaluated as a mechanism in human studies. In T_H2 conditions such as asthma, IL-4 stimulates proliferation of B cells that can act as APC for the development of primary $CD4^+$ T-cell responses.⁵⁵ Reduction in B cells through peptide immunotherapy may also have indirectly diminished T-cell-driven inflammation, thereby contributing to the amelioration of AHR and lung inflammation.

It has been demonstrated previously that generation of allergen-specific Treg cells and increased production of IL-10 regulate allergic responses following specific immunotherapy.⁵⁶ Subjects with allergic asthma, rheumatoid arthritis, and insulin-dependent diabetes treated with allergen peptides, heat shock protein peptides, or pro-insulin peptides, respectively, demonstrated increased levels of IL-10 production in antigen-stimulated PBMC in culture.^{26,33,34} In addition to increased IL-10 production in PBMC culture,²⁶ our previous studies of peptide immunotherapy in cat-allergic mice have also demonstrated increased levels of IL-10 in BAL fluid and IL-10⁺ cells in lung tissue.⁵ In the present study, we also found a marked increase in IL-10 level in lung tissue digest supernatant, and IL-10⁺ cells in both lung tissue and lung-associated DLN. Further analysis of IL-10⁺ cells demonstrated an increased proportion of Fox p3⁺ $CD4^+$ T cells in the IL-10⁺ population from peptide-treated mice. In addition, the mean fluorescence intensity of Fox p3 staining increased, indicating increased levels of Fox p3 within individual cells. However, there was no significant difference in Fox p3⁺ $CD4^+$ cell numbers between vehicle- and peptide-treated mice, in agreement with our earlier studies.⁵ This may be due to transient induction in Fox p3 expression in activated effector cells in vehicle-treated mice.⁵⁷

IL-35 is a recently described member of the IL-12 family of cytokines that has a critical role in the development and suppressive function of Treg.^{58,59} The ability of IL-35 to convert conventional human T cells into IL-35-secreting iTreg cells suggests that these cells may contribute to infectious tolerance.⁵⁹ Recently, in a murine model of OVA-induced allergic airway disease, Whitehead *et al.*⁶⁰ demonstrated that repeated oropharyngeal administration of OVA with lipopolysaccharide resulted in loss of AHR that was dependent on expansion of a population of Treg secreting IL-10, TGF β , and IL-35. Loss of AHR was shown to be dependent on IL-35 alone. To evaluate the contribution of IL-35 to the tolerance observed in our model, we measured the cytokine in BAL, lung tissue digests, and serum. In contrast to IL-10, IL-35 levels did not change after peptide treatment, suggesting that in this particular form

of immunological tolerance, IL-35 does not have a major role in our model of allergic airway disease. Our model was primarily eosinophilic in nature, in contrast to that of Whitehead *et al.*,⁶⁰ which was neutrophilic. An additional important difference between the models may have been the aerosolization of allergen in the neutrophilic model, which may have resulted in oral exposure to the allergen after grooming.

Apart from $CD4^+$ cells, other cells such as B cells, dendritic cells, and macrophages can also produce IL-10. B cells producing IL-10 have been shown to prevent allergic airway inflammation via inducing pulmonary infiltration of Fox p3⁺ $CD25^+$ $CD4^+$ regulatory T cells.^{61,62} We also detected IL-10 expression in B cells in allergic mice; however, at the time point selected for outcome measurements in this model, these cells did not demonstrate increased expression of IL-10 after peptide treatment. Similarly, we also detected very small percentages of IL-10-producing $CD11b^+$ and $CD11c^+$ cells in allergic mice, which did not change after peptide treatment. Interferon- γ that is produced by T_H1 cells is generally considered to antagonize T_H2 responses and exerts inhibitory effects on T_H2 -cell differentiation. In our study, interferon- γ -producing cell numbers in the lung did not increase after peptide treatment. This suggests that peptide therapy did not result in deviation from a T_H2 to a T_H1 response.

In conclusion, our data indicate that peptide immunotherapy can, under the appropriate conditions, modulate established allergic responses to a third-party immunogen. Treatment with two peptides containing $CD4$ T-cell epitopes from the HDM allergen Der p 1 reduced AHR and T_H2 immune responses induced by OVA airway challenge. Amelioration of allergic responses was associated with reduction in OVA-specific T-cell proliferation, the induction of IL-10 and Fox p3, together with a reduction in T_H2 -cell numbers, IL-5-, and IL-17-producing cells. Levels of IL-35 remained unchanged. Exploiting immunological tolerance induced by treatment with a limited number of T-cell epitopes may be important in the potentiation of clinical responses to peptide (and other forms of) immunotherapy in allergic, autoimmune, and transplantation-related diseases. The results of this study will inform future clinical study design.

METHODS

Animals. Female BALB/c mice (8–10 wk of age) were purchased from Charles River (Montreal, QC, Canada), housed in specific pathogen-free conditions and allowed to acclimatize for 1 week before experimental use. All procedures were carried out in accordance with the Guide for the Humane Use and Care of Laboratory Animals and were approved by the Animal Research Ethics Board at McMaster University.

Peptides. For *in vitro* and *in vivo* studies, Der p 1 peptides were synthesized by standard Fmoc chemistry, purified (>95%) by HPLC, and presented as lyophilized solid (GL Biochem, Shanghai, China). Peptide sequences were Der p 1^{55–62}: RNQSLDLAEQELYDSASQH and Der p 1^{149–167}: DEFKNRFLMSAEAFE (amino acid numbering including Der p 1 pro-peptide but exclusive of signal sequence). Lyophilized peptides (10 mg) were reconstituted in a small volume of 10^{-4} M HCl, dilution to 1 mg/ml⁻¹ in phosphate-buffered saline (PBS) and frozen storage (-80°C), before use.

Figure 7 Schematic protocol flow chart for allergen-induced airway disease and treatment with Der p 1 peptides. Airway inflammation was induced in BALB/c mice sensitized intraperitoneally (i.p.) with 10 μ g ovalbumin (OVA) in alum (d 0 and 11) followed by inhalation of 1.5 μ g house dust mite (HDM) (days 22–33 and 36–40) and 100 μ g OVA (days 36 and 37). To induce tolerance, a mixture of HDM peptides Der p 1^{55–69} and Der p 1^{149–167} (1 μ g each) was administered intranasally (i.n.) for 5 consecutive days (days 50–54). After rechallenge with 100 μ g OVA (days 55 and 56), lung function was analyzed and the mice were killed (day 58) for tissue harvest.

Allergen sensitization and peptide treatment. Allergic airway disease was induced in mice by sensitization to OVA (Sigma-Aldrich, St. Louis, MO), followed by inhaled challenges with HDM extract from *Der-matophagoides pteronyssinus* (Greer Laboratories, Lenoir, NC) and OVA to localize inflammation to the lung tissues (**Figure 7**). Mice were sensitized by intraperitoneal injections of 10 μ g OVA in 50 μ l PBS with 150 μ l of alum (Au-Gel-S; Serva Electrophoresis, Heidelberg, Germany) on days 0 and 11. Mice were challenged i.n. with HDM (1.5 μ g protein weight in 25 μ l PBS) on days 22–26, 29–33, and 36–40 and OVA (100 μ g in 25 μ l PBS) on days 11, 36, and 37, during light anesthesia (isoflurane). To induce tolerance in mice with established airway disease, a mixture of Der p 1^{55–69} and Der p 1^{149–167} peptides (1 μ g each in 25 μ l PBS) was administered i.n. for 5 days (days 50–54). Mice were recall challenged i.n. with OVA (100 μ g in 25 μ l PBS) on days 55–56. Forty eight hours after the last challenge, AHR was measured, and BAL fluid, lung tissue, and DLNs were collected, and mice were euthanized via exsanguination.

Measurement of AHR. Airway responsiveness was measured using the *FlexiVent* small animal ventilator system. The single compartment model was used, and total respiratory system resistance (R_{RS}) in response to nebulized methacholine (MCh) was assessed. Mice were anaesthetized by an intraperitoneal injection of xylazine hydrochloride (Bayer, Toronto, ON; 10 mg kg⁻¹) and sodium pentobarbital (Ceva Sante Animale, Lenexa, KS; 30 mg kg⁻¹). A tracheotomy was performed, into which a blunted 18-gauge needle was inserted and secured. The other end of the needle was inserted into the Y-adaptor of the *FlexiVent* apparatus. Mechanical ventilation was commenced at a rate of 150 breaths per minute with a volume of 10 ml kg⁻¹. Pancuronium bromide (Santoz, Boucherville, QC; 20 mg kg⁻¹) was administered intraperitoneally to achieve paralysis and prevent respiratory effort during measurement. Sequential increasing doses (0, 3.1, 6.3, 12.5, and 25 mg ml⁻¹) of MCh were nebulized for inhalation by each mouse, and the resultant R_{RS} was determined through 13 0.4-s perturbations over a 3-min period. Before each dose of MCh, an inflation to total lung capacity was initiated to normalize the data. A dose–response curve to MCh was generated. Heart rate and oxygen saturation were monitored throughout the procedure using a Biox 3700 infrared pulse oximeter (Ohmeda, Boulder, CO) with the probe placed on hind limb of the mouse.

Bronchoalveolar lavage. Immediately after measurement of AHR, mice were killed and the airways were lavaged twice with 0.25 ml PBS as previously described.¹⁵ BAL fluids were centrifuged at 150 g for 10 min, and the supernatants were stored at –20 °C for cytokine analysis in future. Cell pellets were suspended in PBS and total cells enumerated. BAL cell isolates were diluted to an approximate concentration of 5 $\times$ 10⁵ per ml and transferred to slides by cytocentrifugation. The cells were Wright–Giemsa stained and differentiated by morphological criteria as one of the following: eosinophil, neutrophil, macrophage, and lymphocytes. Two slides of 200 cells per slide were differentiated by a blinded investigator, and the relative proportions of each cell type were determined and multiplied by the total number of BAL cells obtained to determine absolute cell counts.

Lung cell isolation. After perfusion with PBS containing heparin (10 U ml⁻¹), the right lung was harvested, diced, and incubated on an

orbital shaker for 90 min at 37 °C in digestion medium (Sigma-Aldrich, RPMI-1640 medium containing 10% fetal bovine serum; Invitrogen, Burlington, ON, Canada; 300 U ml⁻¹ collagenase type 1; Worthington Biochemical Corporation (Lakewood, NJ), 50 U ml⁻¹ DNase; Sigma-Aldrich, 100 U ml⁻¹ penicillin; Invitrogen, and 100 μ g ml⁻¹ streptomycin; Invitrogen). After vortexing for 10–15 s, the product of digestion was passed through 70 μ m cell strainer (BD Falcon, Franklin Lakes, NJ). Supernatant collected after centrifugation was stored at –80 °C for later cytokine analysis, and the cell pellet was resuspended. Cells were further purified by histopaque density gradient (histopaque-1083; Sigma-Aldrich).

DLN cell isolation. Mediastinal and peribronchial lymph nodes were removed, and cells were dissociated by applying gentle pressure with a syringe plunger over a 40- μ m cell strainer. After washing twice, the cells were suspended in complete culture medium (RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 μ M 2-mercaptoethanol, 100 U ml⁻¹ penicillin, and 100 μ g ml⁻¹ streptomycin).

Histology. Left lungs were inflated with 10% buffered formalin to a pressure of 20 cm H₂O, fixed for at least 24 h, and following dehydration paraffin-embedded. Sections of 3 μ m were stained with hematoxylin and eosin and periodic acid-Schiff to quantify eosinophils and mucin-containing goblet cells, respectively. Sections were examined under light microscopy in a blinded fashion, as described previously.⁶³

Flow cytometry. Cells were pre-incubated with anti-CD16/32 monoclonal antibody (2.4G2; BD Biosciences, San Jose, CA) to block Fc γ R. For surface marker staining, Fc γ R-blocked cells were incubated with PE-anti-CD4 (RM4-5; BD Biosciences), PerCp-anti-CD8 (53-6.7; BD Biosciences), APC and PE-anti-CD19 (1D3; BD Biosciences), and FITC-anti-T1/ST2 (DJ8; MD Biosciences) monoclonal antibody for 30 min at 4 °C. After washing, cells were resuspended in 1% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA).

For intracellular cytokine staining, cells were stimulated in 24-well plates with phorbol 12-myristate 13-acetate (20 ng ml⁻¹; Sigma-Aldrich), ionomycin (1 mM; Sigma-Aldrich), and brefeldin A (10 μ g ml⁻¹; Sigma-Aldrich) for 6 h at 37 °C. Cells were stained for surface markers as described above, fixed with 4% paraformaldehyde, and permeabilized in Perm/Wash buffer (BD Biosciences) before intracellular staining with anti-cytokine monoclonal antibody. Cells were stained for intracellular cytokines by incubating the cells in Perm/Wash buffer containing a predetermined optimal concentration of APC-anti-IL-10 (JES5-16E3; BD Biosciences), APC-anti-IL-17A (ebio17B7; eBiosciences, San Diego, CA), and APC-anti-IL-5 (TRFK5; BD Biosciences) monoclonal antibody for 30 min at 4 °C.

Intracellular Fox p3 staining was performed using mouse regulatory T-cell staining kit (88-8111; eBiosciences) according to the manufacturer's instructions. Flow-cytometry analysis was performed using FACSCanto II (BD Biosciences) and Flowjo software (TreeStar, Ashland, OR).

Measurement of IL-10 protein levels. Levels of IL-10 present in BAL fluids and lung tissue digest supernatants were measured using an

ELISA kit (sensitivity, 15 pg ml⁻¹; eBiosciences) according to the manufacturer's instructions.

Measurement of IL-35 protein levels. Levels of IL-35 present in BAL fluids, lung tissue digest supernatants, and serum were measured using an ELISA kit (sensitivity, 5.7 pg ml⁻¹; USCN Life Science, Wuhan, China) according to the manufacturer's instructions.

Cell proliferation. Proliferation assays of cells isolated from lung-associated DLN were performed with [³H] thymidine and CFSE. For the [³H] thymidine assay, cells were cultured in triplicate at 2 × 10⁴ cells per well, in U-bottom 96-well plates, in the presence of OVA, HDM, or complete medium alone at 37 °C in 5% CO₂ in air. After 6 days of culture, 0.5 µCi [³H] thymidine per well was added, and the cells were cultured for another 18 h. [³H] thymidine incorporation data in counts per minute (cpm) was converted into stimulation index by dividing cpm obtained from stimulated cells by cpm values from unstimulated wells. CFSE assay of proliferation was performed to identify the CD4 T-cell proliferation. Cells were stained with CFSE (CellTrace CFSE Cell Proliferation Kit; Invitrogen) at the final concentration of 1 µM as described previously¹⁷. CFSE-stained cells were cultured at 4 × 10⁵ cells per well in flat-bottom 96-well plate in the presence of 20 µg ml⁻¹ OVA, HDM, or medium alone at 37 °C in 5% CO₂ in air. After 6 days, cells were harvested and surface stained with PE-anti-CD4 antibody as described previously. Flow-cytometry analysis was performed using a FACSCanto II cytometer, and data were analyzed with FlowJo software.

Statistical analysis. Data were expressed as mean ± s.e.m. and analyzed for statistical significance using Student's *t*-tests. Differences were considered statistically significant at a *P*-value ≤ 0.05.

ACKNOWLEDGEMENTS

This study was supported by the Canadian Institutes for Health Research (CIHR). M.L. is supported by the Canada Research Chairs Program, the Canada Foundation for Innovation, and the McMaster University/GSK Chair in Lung Immunology at St. Joseph's Healthcare. D.M.M. is supported by a Father Sean O'Sullivan Research Committee (FSORC) Post-graduate Studentship through St. Joseph's Healthcare, Hamilton. M.S.B. is supported through an award to M.L. from FSORC and the Department of Medicine, McMaster University. C.M.L. is a Wellcome Trust Senior Research Fellow.

DISCLOSURE

M. Larché is a founder, stockholder, and consultant of Circassia Ltd. and a founding scientist of Adiga Life Sciences Inc. and has received research support from both of these companies. The remaining authors declared no conflict of interest.

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