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Antigen-Fixed Leukocytes Tolerize Th2 Responses in Mouse Models of Allergy

Charles B. Smarr,*† C.-L. Hsu,*†† Adam J. Byrne,‡ Stephen D. Miller,*† and Paul J. Bryce*‡

Allergic diseases, including asthma and food allergies, are increasing health concerns. Immunotherapy is an effective therapeutic approach for many allergic diseases but requires long dose escalation periods and has a high risk of adverse reactions, particularly in food allergy. New methods to safely induce Ag-specific tolerance could improve the clinical approach to allergic disease. We hypothesized that Ag-specific tolerance induced by the i.v. injection of Ags attached to the surface of syngeneic splenic leukocytes (Ag-coupled splenocytes [Ag-SPs]) with the chemical cross-linking agent ethylene-carbodiimide, which effectively modulate Th1/Th17 diseases, may also safely and efficiently induce tolerance in Th2-mediated mouse models of allergic asthma and food allergy. Mice were tolerized with Ag-SP before or after initiation of OVA/alum-induced allergic airway inflammation or peanut-induced food allergy. The effects on disease pathology and Th2-directed cytokine and Ab responses were studied. Ag-SP tolerance prevented disease development in both models and safely tolerized T cell responses in an Ag-specific manner in presensitized animals. Prophylactically, Ag-SP efficiently decreased local and systemic Th2 responses, eosinophilia, and Ag-specific IgE. Interestingly, Ag-SP induced Th2 tolerance was found to be partially dependent on the function of CD25+ regulatory T cells in the food allergy model, but was regulatory T cell independent in the model of allergic airway inflammation. We demonstrate that Ag-SP tolerance can be rapidly, safely, and efficiently induced in murine models of allergic disease, highlighting a potential new Ag-specific tolerance immunotherapy for Th2-associated allergic diseases. The Journal of Immunology, 2011, 187: 5090–5098.

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Abbreviations used in this article: Ag-SP, Ag-coupled splenocyte; BAL, bronchoalveolar lavage; CBA, cytometric bead array; EAE, experimental autoimmune encephalomyelitis; ECDI, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; MBP, myelin basic protein; mMCP-1, mouse mast cell protease 1; OVA-SP, ovalbumin-coupled splenocyte; PAS, period acid-Schiff; SEB, staphylococcal enterotoxin B; Treg, regulatory T cell; WPE, whole peanut extract; WPE-SP, WPE-coupled splenocyte.
clinical trial is being performed by Dr. S. Miller in collaboration with Dr. Roland Martin (University of Hamburg, Hamburg, Germany) (EudraCT no. 2008-004408-29) to examine the safety and efficacy of tolerance to a mixture of myelin-depleted peptides ECDI-fixed to autologous cells for treatment of new-onset multiple sclerosis.

In this study, we used two murine models of allergy, a food allergy model to whole peanut extract (WPE) (16) and an OVA-induced allergic airway inflammation model (17) to investigate the efficacy of Ag-SP–induced tolerance to regulate allergic responses. In each model, Ag-SP tolerance prevented disease development and tolerated T cell responses in an Ag-specific manner in presensitized animals. Prophylactically, Ag-SP decreased local and systemic Th2 responses, eosinophilia, and Ag-specific IgE. In addition, short-term induction of Ag-SP–induced tolerance was found to be independent of the function of CD25+ Tregs in the allergic airway inflammation, but partially dependent on Tregs in the food allergy model. The current experiments demonstrate that Ag-SP tolerance can be induced rapidly, safely, and efficiently in murine models of Th2-associated disease, highlighting a potential new Ag-specific immunotherapy approach.

Materials and Methods

Animals

Four- to 8 wk-old C3H/HeJ (The Jackson Laboratory, Bar Harbor, ME) and BALB/c mice (Taconic Farms, Hudson, NY) were maintained in specific pathogen-free conditions at Northwestern University Center for Comparative Medicine. C3H/HeJ mice were maintained on a peanut-free diet. All protocols were approved by the Northwestern University Animal Care and Use Committee.

Reagents

PC61 Ab (anti-CD25, rlgG1) and isotype control (rlgG1) were purchased from Bio X Cell (West Lebanon, NH). Treg depletion–CD25 receptor downregulation was assessed by staining with 7D4 Ab (anti-CD25, rlgM) (18). WPE was prepared from unsalted, uncooked peanuts, as described previously (19). Staphylococcal enterotoxin B (SEB; Toxin Technology, Sarasota, FL), covered under federal select agents requirements, was used as approved by the Northwestern University Office of Research Safety under the requirements of the Centers for Disease Control and Prevention and the Department of Agriculture and according to necessary containment and use reporting mandates.

Food allergy model

Murine food allergy model was performed as described previously (16). Mice were given 50 µg SEB with or without 100 µg WPE by oral gavage once per week for 8 wk. Mice were challenged orally with 5 mg WPE on week 9. Symptom scores and body temperature were determined after challenge as described previously (20, 21). A score of 0 was assigned if no symptoms were evident; 1 represents mild scratching, rubbing, or both of the nose, head, or feet; 2 and 3 represent intermediate symptoms (e.g., edema around the eyes or mouth, pilar erection, labored breathing); 4 represents significantly reduced motility, tremors, and significant respiratory distress; and 5 represents death. Symptom scores were determined by two investigators who were blinded to the experimental conditions.

Allergic airway inflammation model

Mice were immunized i.p. with two doses of 10 µg OVA (grade V; Sigma-Aldrich, St. Louis, MO) in alum (3 mg) or alum and PBS alone. Mice were challenged for 20 min with aerosolized OVA (10 mg/ml) for three consecutive days prior to tissue harvest (20).

Ag-coupled cell tolerance

Tolerance was induced by i.v. injection of ECDI-fixed, Ag-coupled splenocytes (Ag-SP), as described previously (10). Splenocytes from syngeneic mice were coupled to OVA, WPE, myelin basic protein (MBP) (in all allergic airway inflammation studies), or BSA (in all food allergy model studies; 1 mg/ml) via incubation with ECDI (150 mg per 3.2 × 10^6 cells; Calbiochem, San Diego, CA) for 1 h at 4°C followed by washing; 5 × 10^7 Ag-SP in 200 µl PBS were injected i.v. per mouse.

Analysis of bronchoalveolar lavage and peripheral blood cells

Lungs were flushed with bronchoalveolar lavage (BAL) fluid (1 mM EDTA and 10% FCS in PBS). Total cell counts were determined and samples were subjected to cytofluorimetry onto slides and DiffQuik (Siemens, Newark, DE) stained for differential cell counts (21). Blood was collected into EDTA-coated tubes and stained with Discombe’s fluid, and eosinophils were counted using a hemocytometer (22).

Histology

Tissues were collected, fixed in formalin, embedded in paraffin, and stained with H&E or periodic acid-Schiff (PAS) by Histo-Scientific Research Laboratories (Mount Jackson, VA).

Ag-specific Abs

Serum was collected upon sacrifice, and tissue harvest and Ag-specific Abs were quantified by sandwich ELISA as described previously (16).

OVA-specific recall responses

Mediastinal lymph nodes were harvested and incubated with OVA in serum-free HL-1 culture medium at 37°C for 48 h and pulsed with 1 µCi/well ['^3H]Tdr for the last 24 h of culture. Proliferation was determined by ['^3H]Tdr incorporation as detected by a Topcount Microplate Scintillation Counter.

WPE-specific recall responses

Spleens were harvested and incubated with 100 µg/ml WPE in complete RPMI-1640 at 37°C for 72 h.

Cytokine quantification

Cell-free BAL fluid and supernatants from recall response cultures were assayed for production of IL-4, IL-5, IL-13, and IFN-γ by cytometric bead array (CBA; BD Biosciences, San Jose, CA), Milliplex (Millipore, Billerica, MA), or ELISA. Serum mouse mast cell protease 1 (mMCP-1) was quantified by mMCP-1 ELISA kit (eBioscience, San Diego, CA).

Statistics

Statistics were performed on GraphPad Prism 5 software (GraphPad Software, La Jolla, CA) using Student t test or one-way ANOVA to determine significance.

Results

Ag-SP tolerance prevents induction of allergic disease

We investigated the ability of Ag-SP tolerance to prevent allergic responses by pretreating mice with ECDI-fixed Ag-coupled splenocytes in two murine models of allergy: an OVA-induced allergic airway inflammation model and a food allergy model to WPE. In the allergic airway inflammation model, we administered OVA-coupled splenocytes (OVA-SP) prior to each of two sensitizations with OVA in alum adjuvant (Fig. 1A). We first examined the effect of OVA-SP as compared with Sham-SP (MBP-SP) on local allergic responses induced by an inhalation challenge of aerosolized OVA. Analysis of BAL fluid showed that OVA-SP–treated mice had significantly reduced lung eosinophilia (Fig. 1B). In addition, the Th2-associated cytokines IL-4 and IL-5 were significantly reduced in the BAL fluid of OVA-SP–treated mice compared with Sham-SP (MBP-SP) on local allergic responses induced by an inhalation challenge of aerosolized OVA. Analysis of BAL fluid showed that OVA-SP–treated mice had significantly reduced lung eosinophilia (Fig. 1B). In addition, the Th2-associated cytokines IL-4 and IL-5 were significantly reduced in the BAL fluid of OVA-SP–treated mice. The systemic allergic response was also inhibited, as blood eosinophil concentrations were reduced (Fig. 1D) and serum OVA-specific IgE levels were also dramatically reduced (Fig. 1E) in OVA-SP–treated mice.

In the food allergy model, a model with a strong mast cell–mediated anaphylactic component, we administered WPE-coupled splenocytes (WPE-SPs) prior to sensitization (Fig. 2A). Fifteen minutes after oral challenge with WPE, mice tolerized with WPE-SP showed significantly decreased symptom scores compared with Sham-SP tolerized mice (Fig. 2B). Furthermore, core body temperature was also measured after challenge because systemic anaphylaxis leads to vascular leak and hypothermia as a consequence of hypotension. Serum levels of mMCP-1, a major pro-
tease released following mast cell activation, were measured after challenge as well. We found that WPE-SPs significantly prevented core temperature decreases (Fig. 2C) and mMCP-1 release into serum (Fig. 2D) compared with Sham-SP controls. This finding indicated that WPE-SPs prevent WPE-specific anaphylaxis in this model. As in the allergic airway inflammation model, WPE-SP tolerization also significantly reduced systemic allergic responses. Peripheral blood eosinophil numbers were significantly reduced

**FIGURE 1.** OVA-SP tolerance specifically inhibits Th2 responses in OVA/alum immunized mice. A, BALB/c mice (n = 5) were tolerized i.v. with OVA-SP or Sham MBP-SP, sensitized with OVA/alum and challenged as shown. B, BAL eosinophils were quantified by differential staining. C, IL-4 and IL-5 in BAL fluid were quantified by Milliplex. D, Peripheral blood eosinophils were quantified. E, Serum OVA-specific IgE was quantified by ELISA. F and G, Mediastinal lymphocytes were restimulated with 25 μg/ml OVA; proliferation was quantified by [3H]TdR incorporation (F); and IL-4, IL-5, and IL-13 were quantified by CBA (G). Results are mean ± SEM and representative of three experiments. ***p < 0.001.

**FIGURE 2.** WPE-SP tolerance specifically inhibits disease symptoms and Th2 responses in a food allergy model. A, C3H/HeJ mice (n = 7–10) were tolerized i.v. with WPE-SP or Sham (BSA-SP), sensitized and challenged with WPE as shown. B, Fifteen minutes after challenge, clinical symptoms were scored and (C) mouse body temperature was measured for 1 h. D, Serum mMCP-1 collected 24 h after challenge was quantified by ELISA. E, Peripheral blood eosinophils were quantified. F, Serum WPE-specific IgE and IgG1 and (G) IL-4, IL-5, IL-13, and IFN-γ production from splenocytes restimulated with 100 μg WPE were quantified by ELISA. Results are means ± SEM from three experiments. *p < 0.05, **p < 0.01, ***p < 0.001. n.d., not detectable.
(Fig. 2E), and serum WPE-specific IgE was undetectable in WPE-SP–treated mice whereas WPE-specific IgG1 was unchanged (Fig. 2F).

These results clearly demonstrate that, in addition to inhibiting Th1 and Th17 associated responses as published previously, Ag-SP tolerance can also prevent the induction of Ag-specific, Th2-associated allergic diseases.

Ag-SP tolerize Ag-specific Th2 cytokine responses

In the OVA-induced allergic airway inflammation model, restimulation of cells from mediastinal lymph nodes demonstrated that OVA-SP effectively tolerized OVA-specific T cells and prevented the induction of a Th2 response. Following OVA restimulation, lymphocytes from OVA-SP treated mice had significantly reduced proliferation compared with Sham-SP controls (Fig. 1F). These cells also produced significantly lower levels of IL-4, IL-5, and IL-13 upon OVA restimulation (Fig. 1G), with no effect on IFN-γ (data not shown). Similar data were seen after WPE restimulation of splenocytes from WPE-SP tolerized mice compared with Sham-SP controls in the food allergy model (Fig. 2G). These results indicate that Ag-SP tolerization inhibits the induction of Th2 cytokine responses in an Ag-specific manner.

Protective tolerance induced by Ag-SP is Treg independent in OVA-induced allergic airway inflammation, but Tregs contribute to tolerance in peanut-induced food allergy

Our previous work in a model of allogeneic islet transplant rejection indicated an important role for Tregs in the induction of tolerance by Ag-SP (9). We investigated whether Tregs were also involved in the induction of tolerance by Ag-SP in the two murine models of allergic disease by treating mice with PC61 to functionally inactivate Tregs; using a treatment regimen similar to what we established previously will alter Ag-SP responses in EAE (18). In the OVA-induced allergic airway inflammation model, we injected mice with PC61 around the time of tolerance (Fig. 3A). PC61-treated mice showed a trend of exacerbated allergic airway inflammation, as has been reported previously (23). However, OVA-SP tolerance reduced local inflammation in PC61-treated mice as effectively as in isotype control Ig-treated mice, because eosinophils in the BAL fluid of PC61-treated mice were reduced by OVA-SP (Fig. 3B), indicating a Treg-independent mechanism of tolerance induction. In addition, IL-4, IL-5, and IL-13 were also significantly reduced by OVA-SP in the BAL fluid of PC61-treated animals (Fig. 3C). Histologic analysis of lung sections indicated that OVA-SP reduced cellular infiltrate and mucus secretion as shown by H&E and PAS staining, respectively (Fig. 4). Inhibition of the sensitization response by OVA-SP was also Treg independent because OVA-SP significantly reduced serum OVA-specific IgE in PC61-treated animals (Fig. 3D). Restimulation of cells from mediastinal lymph nodes further demonstrated that OVA-SP induced tolerance of OVA-specific T cells in a Treg-independent manner. In PC61 treated mice, OVA-SP reduced proliferation following OVA restimulation of lymphocytes (Fig. 3E). These cells also produced significantly less IL-4, IL-5, and IL-13 upon OVA restimulation (Fig. 3F). Conversely, in the food allergy model, in which we injected mice with PC61 shortly after the induction of tolerance (Fig. 5A), mice that received PC61 had significantly higher symptom scores (Fig. 5B) and, whereas one mouse exhibited only a modest decrease in body temperature, the other two mice tested had robust decreases in body temperature.
WPE-specific IgE was quantified by ELISA (n = 3). The figure shows that WPE-specific IgE (Fig. 5C) was not quite statistically significant. However, serum WPE-specific IgE (Fig. 5B) versus isotype-treated controls, although not quite statistically significant. However, serum WPE-specific IgE (Fig. 5F) and IgG1 (data not shown) and IL-4, IL-5, and IL-13 produced from WPE-restimulated splenocytes (data not shown) were unaffected by Treg inactivation compared with isotype-treated controls. While the symptom scores between sham-treated (BSA-SP) and isotype-treated controls also failed to reach significance (p = 0.08 by unpaired t test), in all other readouts these groups were significantly different, reflective of our similar findings in Fig. 2. Collectively, these results indicate that Tregs are partially responsible for the induction of tolerance by Ag-SP in the WPE-induced food allergy model, but not in an allergic OVA-induced airway inflammation model and imply that Tregs are one of several mechanisms of tolerance induced by Ag-SP that control the allergic effector response. These results also support the mechanisms of allergic responses being different between models of airway disease and food allergy, with food allergy exhibiting a stronger dependence on Treg-mediated tolerance.

**FIGURE 4.** OVA-SP tolerance inhibits OVA-induced airway inflammation in a Treg-independent fashion. Mice were treated as indicated in Fig. 3A. Representative histology sections from mice sacrificed at day 35, stained with H&E or PAS as indicated, are shown at ×200 original magnification.

Mice receiving PC61 also exhibited a trend toward enhanced mMCP-1 in the serum (Fig. 5D) and increased peripheral blood eosinophilia (Fig. 5E) versus isotype-treated controls, although not quite statistically significant. However, serum WPE-specific IgE (Fig. 5F) and IgG1 (data not shown) and IL-4, IL-5, and IL-13 produced from WPE-restimulated splenocytes (data not shown) were unaffected by Treg inactivation compared with isotype-treated controls. While the symptom scores between sham-treated (BSA-SP) and isotype-treated controls also failed to reach significance (p = 0.08 by unpaired t test), in all other readouts these groups were significantly different, reflective of our similar findings in Fig. 2. Collectively, these results indicate that Tregs are partially responsible for the induction of tolerance by Ag-SP in the WPE-induced food allergy model, but not in an allergic OVA-induced airway inflammation model and imply that Tregs are one of several mechanisms of tolerance induced by Ag-SP that control the allergic effector response. These results also support the mechanisms of allergic responses being different between models of airway disease and food allergy, with food allergy exhibiting a stronger dependence on Treg-mediated tolerance.

**FIGURE 5.** WPE-SP-induced tolerance is partially Treg dependent. A, C3H/HeJ mice were tolerized, sensitized, and challenged as shown. Mice were treated with 1 mg PC61 or isotype at day −2. B, Symptom scores were determined 30 min after challenge (n = 7, pooled from 3 independent experiments). C, Mouse body temperature (n = 3 per group, measured during one experiment from B) and (D) serum mMCP-1 (n = 4–5, measured during one experiment from B) were determined after challenge. E, Peripheral blood eosinophils were quantified (n = 4–5, measured during one experiment from B). F, Serum WPE-specific IgE was quantified by ELISA (n = 8–9, pooled from two independent experiments). Results are mean ± SEM from three experiments. *p < 0.05, **p < 0.01 by unpaired t test. n.d., not detectable.

**Treatment with Ag-SP tolerizes the Ag-specific T cell response after disease onset**

The primary clinical interest in allergic immunotherapy is in treating established hypersensitivity in an Ag-specific manner. Previous work in models of autoimmune disease has shown Ag-SP to be capable of inducing tolerance for amelioration of ongoing disease. Importantly, as anaphylaxis remains a major concern of immunotherapy therapy for food allergy, the inability of Ag-SP to trigger anaphylactic responses in sensitized mice has been established in autoimmune models of disease (14). We therefore sought to determine whether Ag-SP could be similarly useful in a therapeutic context in allergic disease. To this end, we administered Ag-SP after sensitization in the OVA-induced allergic airway inflammation model (Fig. 6A) and the food allergy model (Fig. 7A). In both models, Ag-SP treatment demonstrated a significant reduction of most of the Th2-mediated cytokine release. Analysis of cytokine concentrations in the BAL fluid from the allergic airway sensitization model showed a significant reduction in IL-5, a reduction in IL-4, but no change in IL-13 of OVA-SP treated mice (Fig. 6C). Restimulation of cells from mediastinal lymph nodes in this model further demonstrated that, administered after sensitization, OVA-SP tolerized the T cell response to OVA because lymphocytes from OVA-SP treated animals had significantly reduced proliferation to OVA restimulation compared with Sham-SP treated controls (Fig. 6E). These cells also produced significantly less IL-4, IL-5, and IL-13 upon OVA restimulation (Fig. 6E), demonstrating successful tolerization of Th2 cells in presensitized mice. Similarly, WPE restimulation of splenocytes from WPE-SP tolerized mice in the food allergy model produced significantly less IL-4, IL-5, and IL-13 than seen in Sham-SP or soluble WPE controls (Fig. 7H). In response to tolerance induction, mice previously sensitized with WPE showed no anaphylactic symptoms and less mMCP-1 in serum, while those receiving splenocytes with soluble WPE developed significant anaphylactic responses within 30 min (Fig. 7B) and higher mMCP-1 within 1 h (Fig. 7C). Upon subsequent challenge with WPE, mice tolerated
with WPE-SP had only modestly lower symptom scores (Fig. 7D) and serum levels of mMCP-1 (Fig. 7E) when compared with Sham-SP controls. Peripheral blood eosinophil counts were also significantly lower in WPE-SP tolerized mice 24 h after challenge (Fig. 7F). Surprisingly, there was no correlative reduction in, and sometimes higher levels of, systemic Ag-specific IgE levels in both models (Figs. 6D, 7G) as well as an increase in BAL fluid lung eosinophilia in the allergic airway inflammation model relative to Sham-SP controls (Fig. 6B). However, WPE-specific IgG1 levels were not altered (data not shown). Despite these findings, these studies indicate that Ag-SP treatment can tolerize Ag-specific Th2 responses and significant reduction of symptom scores in presensitized recipients.

Discussion

The development of efficient, safe means of inducing specific tolerance to Ags is important in allergic disease research because many patients end conventional immunotherapy because of side effects and the long treatment courses, especially patients with a food allergy (24). We, and others, have previously demonstrated the ability of Ag-SP to prevent and treat Th1/Th17-dependent autoimmune disease and transplant rejection (7–9), including showing that Ag-SP tolerance inhibited the induction of allospecific Ab production. To our knowledge, the present study is the first to show the ability of Ag-SP to tolerize Th2 responses. We demonstrate the efficacy of Ag-SP treatment both in the prevention of allergic responses and the tolerization of presensitized Th2-associated responses in two murine models of allergic disease. Our results indicate several key advantages of Ag-SP-induced tolerance over existing specific immunotherapies, including its notable Ag specificity and efficiency, requiring only two treatments with a total of only 30–40 μg of cell-bound Ag (data not shown). In addition, and critically in the context of anaphylaxis, Ag-SP-induced tolerance does not trigger adverse responses when administered to presensitized animals.

We first examined the ability of Ag-SP to prevent the induction of Th2-mediated allergic disease using two different models that share some aspects of immune reactivity but differ in others. In a well-established OVA/alum-induced model of allergic airway inflammation, a strongly T cell-dependent model with little or no mast cell involvement, OVA-SP prevented local allergic inflammation. Recruitment of eosinophils to the lungs was inhibited, as was the production of local Th2 cytokines. In the WPE/SEB-induced food allergy model, a model with Th2-associated responses but in which mast cell-dependent anaphylaxis is a hallmark response, WPE-SP effectively inhibited the anaphylactic symptoms and drops in body temperature seen after oral WPE challenge. In both models, Ag-SP inhibited systemic eosinophilia and Ag-specific IgE. Interestingly, Ag-specific IgG1 (data not shown) was also reduced in the airway inflammation model but not in the food allergy model, perhaps relating to the different routes of immunization and adjuvants used in the two models. Because IgE and IgG1 can induce anaphylactic responses by binding Fc receptors on mast cells (25), the presence of IgG1 in the food allergy model could explain the mild anaphylactic symptoms observed in the WPE-SP tolerized group. Indeed, IgE-deficient mice still exhibit anaphylactic-like reactivity (26). In addition to changes in responsiveness and Abs, T cell cytokine responses were profoundly affected because restimulated lymphocytes failed to proliferate and did not produce Th2 cytokines. Importantly, Th1 or Th17 cytokines were not enhanced (Fig. 2G and data not shown), indicating that Ag-SP induced Ag-specific tolerance to
Th2 responses, rather than skewing responses toward an alternative T helper phenotype. This finding is consistent with our earlier work showing that Ag-SP inhibited Th1/Th17 cytokine production in EAE (27) and suggests that Ag-SP exerts tolerance toward a specific Ag regardless of the type of effector T cell response being generated in that model.

The mechanisms of Ag-SP-induced tolerance have been investigated previously in our Th1/Th17-mediated models of autoimmunity and transplant rejection. Tregs are critical for the induction of tolerance by Ag-SP in a model of alloantigen-specific islet cell transplantation (9), and for long-term tolerance maintenance in the EAE model (11, 27). Using a similar Treg-inhibiting PC61 Ab treatment approach, we show some evidence of Treg dependence of Ag-SP tolerance induction in allergic disease as well, most notably in our mast cell-dependent model of food allergy.

Interestingly, the two models showed significant differences in the dependence of Treg responses. In the OVA-induced model of allergic airway inflammation, tolerance of local inflammation by Ag-SPs was Treg independent, as was inhibition of a Th2 recall response from draining lymph nodes. These results in this acute model of inflammation are consistent with previous observations that the induction of tolerance by Ag-SPs in an acute model of EAE is Treg independent. In autoimmunity, we have shown that Ag-SPs undergo apoptosis and are taken up and represented by host APCs in a tolerogenic fashion (10). In addition, clonal anergy induced by costimulatory blockade as well as negative costimulation by molecules such as PD-1 and CTLA-4 are also important in the induction of tolerance (8, 9, 11–13). These mechanisms may contribute in allergic airway inflammation tolerance; however, we have also found that Tregs were critical for long-term maintenance of tolerance in EAE (11). Therefore, future studies should address the possibility that Tregs, although dispensable in the short-term for the induction of tolerance, may be required for long-term maintenance of tolerance in this model of allergic airway inflammation.

Conversely, in the peanut food allergy model, Ag-SP tolerance is Treg dependent. As anaphylactic symptom scores were restored, reduced body temperature was observed again and slightly higher serum mMCP-1 was detected in Ag-SP–treated mice receiving Treg inactivation. This model of allergy, and these readouts in particular, are mast cell dependent (16), whereas the OVA-induced model of airway inflammation is thought to be relatively mast cell independent (28). Mast cell degranulation releases several mediators that can cause anaphylaxis. Recently, it has been shown that Tregs can inhibit mast cell degranulation via OX40–OX40L interactions (29). Therefore, the enhanced symptom scores seen in PC61-treated mice may be due to the loss of mast cell inhibition by Tregs, but this remains to be determined.

Similar in both models, we observed that inhibition of Ag-specific IgE levels was unchanged by PC61 treatment, indicating that B cell tolerance by Ag-SPs is Treg independent. One potential mechanism for this Treg-independent B cell tolerance is the recognition of Ag in the absence of appropriate CD40 stimulation. CD40/CD40L interactions are required for B cell proliferation and class switching (30). We recently demonstrated that CD4+ T cells isolated from Ag-SP–treated mice fail to upregulate CD40L following in vitro Ag challenge and that treatment with a CD40 agonist abrogates Ag-SP–induced tolerance (31). We hypothesize that, in the absence of CD40L-mediated costimulation from T cells in Ag-SP treated mice, Ag-specific B cells will encounter Ag but fail to undergo class switching, thus accounting for the Treg-independent reduction in IgE production.

Clinically, allergic immunotherapy must induce Ag-specific tolerance in sensitized subjects. A recent trial using an oral immunotherapy treatment for peanut allergy showed that most patients...
(93%) had adverse reactions to the treatment (5). We previously demonstrated that Ag-SP treatment can successfully ameliorate established EAE and that the treatment is safe and not anaphylactic, unlike the administration of soluble Ag to mice with ongoing EAE (14). Crucially in our food allergy model, Ag-SP did not induce anaphylaxis or release of mMCP-1 in sensitized mice. In both models of disease, Ag-SP successfully downregulated the Th2 response in presensitized mice in an Ag-specific manner, as indicated by decreased proliferation and Th2 cytokine production. However, within the time frame we examined, anaphylactic symptoms and serum level of mMCP-1 in response to Ag challenge in the food allergy model were only slightly reduced by Ag-SP treatment. Future experiments will address whether this efficient blockade of Th2 responses might lead to more significant losses in mast cell reactivity if more time or additional treatments with Ag-SP are provided before challenge. Interestingly, blood eosinophilia was suppressed in food allergy model; however, in our allergic airway inflammation model, lung eosinophilia was enhanced by Ag-SP treatment and blood eosinophilia was not significantly affected (data not shown). These differences may be due to the different sensitization protocols in each model. Alternatively, apoptotic Ag-SP traffic to the lungs in large numbers after i.v. injection (S.D. Miller, unpublished results) and may, in such close proximity to aerosolized Ag, be responsible for enhanced eosinophilic inflammation.

In both models, Ag-SP treatment resulted in higher serum concentrations of Ag-specific IgE. This observation mirrors that of a recent oral immunotherapy study in which treatment initially enhanced serum IgE levels (32). However, IgE decreased in the following months. Because of the short duration of our studies, we are currently determining whether a similar decrease in IgE will be observed over time. It is also possible that the increase in Ag-specific IgE is due to presensitized B cells secreting IgE in response to direct or indirect presentation of Ag derived from apoptotic Ag-SP in a T-independent manner. Despite the increased IgE and eosinophilia seen in this study, we do not see a corresponding anaphylaxis response. The profound tolerization of the Ag-specific Th2 response under therapeutic conditions should effectively ameliorate the T cell help required for sustained production of allergic Abs. Indeed, in other models, Ag-SP treatment has been demonstrated to induce long-lasting tolerance (27). Over a sufficient period, as primed B cells turn over and as FcR-bound IgE and IgG1 Abs decay, the lack of help from the tolerized T cell compartment should contribute to amelioration of disease. In addition, Ag-SP treatment would be predicted to be more effective when used in combination with treatments that target non-T cell-dependent arms of the allergic response, such as anti-IgE therapies (3).

Our data establish that, in two models of Th2-associated allergic disease, Ag-SP treatment effectively prevents the induction of disease and safely and specifically tolerizes the Th2 response after onset of disease. Mechanistically, tolerance in the two models is differentially dependent on Tregs, with airway responses being largely unaffected by Treg inactivation, whereas the prevention of mast cell-dependent anaphylaxis to peanut is Treg dependent. Importantly, the ability of Ag-SP to induce tolerance to mixed protein products such as WPE demonstrates its utility to tolerize responses against multiple allergic Ags, similar to our previously published demonstration that tolerization with Ag-SPs linked to mouse spinal cord homogenate can effectively prevent EAE (27, 33, 34). Collectively, our data demonstrate that Ag-SP treatment can induce protective Ag-specific tolerance in models of Th2-mediated immune responses and presents an attractive means of developing a safe and effective therapy to prevent and treat allergic disease.

Disclosures
The authors have no financial conflicts of interest.

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