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A Plant-Based Allergy Vaccine Suppresses Experimental Asthma Via an IFN-γ and CD4$^+$CD45RB^{low} T Cell-Dependent Mechanism

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Allergic asthma is currently considered a chronic airway inflammatory disorder associated with the presence of activated CD4$^+$ Th2-type lymphocytes, eosinophils, and mast cells. Interestingly, therapeutic strategies based on immune deviation and suppression have been shown to successfully attenuate the development of the asthma phenotype. In this investigation, we have for the first time used a genetically modified (GM) plant, narrow leaf lupin (*Lupinus angustifolius L.*), expressing a gene for a potential allergen (sunflower seed albumin) (SSA-lupin) to examine whether a GM plant/food-based vaccine strategy can be used to suppress the development of experimental asthma. We show that oral consumption of SSA-lupin promoted the induction of an Ag-specific IgG2a Ab response. Furthermore, we demonstrate that the plant-based vaccine attenuated the induction of delayed-type hypersensitivity responses and pathological features of experimental asthma (mucus hypersecretion, eosinophilic inflammation, and enhanced bronchial reactivity (airways hyperreactivity). The suppression of experimental asthma by SSA-lupin was associated with the production of CD4$^+$ T cell-derived IFN-γ and IL-10. Furthermore, we show that the specific inhibition of experimental asthma was mediated via CD4$^+$CD45RB^{low} regulatory T cells and IFN-γ. Thus, our data demonstrate that a GM plant-based vaccine can promote a protective immune response and attenuate experimental asthma, suggesting that plant-based vaccines may be potentially therapeutic for the protection against allergic diseases. ***The Journal of Immunology*****, 2003, 171: 2116–2126.**

Allergic asthma is characterized by intermittent and reversible airways obstruction, mucus hypersecretion, inflammation of the airways, and enhanced bronchial reactivity to spasmogenic stimuli (1). Clinical and experimental investigations have shown that CD4$^+$ Th2 lymphocytes through the secretion of an array of cytokines (IL-4, IL-5, IL-6, IL-9, IL-10, IL-13) activate inflammatory and resident effector pathways in the lung and predipose to the pathophysiological features of asthma (1–3). Murine models of experimental asthma have also provided corroborative evidence of the importance of the CD4$^+$ Th2-type cells and Th2-type cytokines in the modulation of allergic airways disease (AAD)[2] and are an established model for understanding the immunoregulation and pathology of asthma (4).

The identification of the role of CD4$^+$ T cells in disease processes has led to the concept that selective inactivation or attenuation of T cell function may be a rational therapeutic strategy for the prevention of asthma (3, 5). Indeed, allergen immunotherapy has been successfully used to attenuate allergic diseases in humans by the modulation of allergen-specific Th2 cell function (6). Other strategies include targeting Th2 cell development by immunization with DNA vaccines encoding allergens or bacterial CpG adjuvant sequences; suppression of Th2 pathways by providing Th1 cytokines such as IFN-γ, IL-12, and IL-18; or infection with the Th1-type promoting bacteria, *Listeria* and *Mycobacterium vaccae* (3, 7–12). More recently, the development of Ag-specific Th2 cell unresponsiveness by mucosal administration of soluble Ag by the oral route (termed oral tolerance) has been used in animal models (13, 14).

Oral tolerance results in the specific suppression of cellular and/or humoral immune responses to the specific Ag and is thought to be mediated by one of three mechanisms: deletion of Ag-reactive cells (clonal deletion), inactivation of Ag-reactive cells (anergy), or suppression of Ag-reactive cells by regulatory cells (immune deviation) (15–18). This has led investigators to hypothesize that development of oral tolerance may suppress the onset of inflammatory disorders, in particular autoimmune diseases. By using experimental models of autoimmune disorders, including Th1-mediated models of experimental autoimmune encephalomyelitis, diabetes, Alzheimer's disease, and atherosclerosis, investigators have demonstrated that oral ingestion of specific Ag before induction of disease can lead to the suppression of disease pathogenesis (15, 19–22). Furthermore, in Th2-mediated diseases, including AAD, oral tolerance has also been shown to abrogate disease (13, 14, 23, 24). Collectively, these findings suggest that the induction of oral tolerance can be used to successfully block CD4$^+$ Th1- and Th2-type immune responses and may be a useful therapeutic approach to suppress immune diseases in humans (15, 25).

Recently, edible transgenic plants containing specific Ags have been proposed to be a possible strategy to induce oral tolerance and promote a protective noninflammatory response (26, 27). Although studies are very limited, consumption of edible transgenic plants expressing specific Ags have been shown to stimulate a

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[2] Abbreviations used in this paper: AAD, allergic airways disease; AHR, airways hyperreactivity; DTH, delayed-type hypersensitivity; GM, genetically modified; HPF, high power field; i.g., intragastric; i.t., intratracheal; MBP, major basic protein; PBLN, peribronchial lymph node; Penh, enhanced pause; SSA, sunflower seed albumin; WT, wild type.

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Th1-type immune response that protects against viral and bacterial infections and autoimmune disease (28–32). To date there are no data on the role of genetically modified (GM) plants in the prevention of $CD4^+$ Th2-type T cell-mediated allergic diseases.

Recently, a member of the sunflower (*Helianthus annuus L.*) seed 2S albumin proteins, sunflower seed albumin (SSA), has been identified as a candidate allergenic protein in sunflower seed hypersensitivity (33, 34). SSA has been shown to contain IgE-binding epitopes and possesses amino acid sequence similarity (34%) with the known allergenic 2S albumin protein from the Brazil nut (33, 34). We have generated a GM narrow leaf lupin (*Lupinus angustifolius L.*), expressing a gene for sunflower seed albumin (SSA-lupin) in their seed. Our aim was to examine whether consumption of a GM plant expressing a potential allergen (GM plant vaccine) could promote oral tolerance and suppress the onset of experimental asthma. We show that oral consumption of SSA-lupin seed meal attenuates mucus hypersecretion, pulmonary eosinophilic inflammation, and airways hyperreactivity (AHR) following subsequent allergen exposure. Importantly, the suppression of experimental asthma was associated with the development of a $CD4^+$ $CD45^{low}$ suppressor T cell population and IFN-γ production. Our findings demonstrate that GM plant-based vaccines expressing potential allergens could function as potential therapeutics for the protection against allergic diseases.

Materials and Methods

Intragastric administration of seed meal from nontransgenic (lupin) and transgenic (SSA-lupin)

Seed meal from GM narrow-leafed lupin expressing SSA protein in the seeds (SSA-lupin) and wild-type narrow leaf lupin (lupin) were generated, as described previously (35). Seeds were ground into fine flour in liquid N_2 using a mortar and pestle. This seed meal was then suspended in PBS (0.166 g/ml), homogenized, sieved through a 70-μm mesh, and stored at −70°C until administration. BALB/c mice were intragastrically (i.g.) administered 250 μl of (~100 mg/ml) SSA-lupin or lupin seed suspension twice per week for 4 wk before experimentation, as described in Fig. 1, *A–C*.

Mice and murine model of experimental asthma

BALB/c wild-type (WT), C57BL/6 WT, IFN-$\gamma^{-/-}$ (SV129/C57BL/6), and IL-$10^{tml-/-}$ (C57BL/6) mice were obtained from specific pathogen-free facilities at the Australian National University. The murine model of experimental asthma is described in Fig. 1*A*. In brief, on day 0, BALB/c mice were primed systemically with an i.p. injection of 50 μg of SSA or OVA in alum (1 mg/ml) dissolved in PBS (final volume 200 μl). On days 14 and 16, the mice were anesthetized with an i.v. injection of 100 μl Saffan solution (1/4 diluted in water). Mice were intubated with a 22-gauge catheter needle, and 25 μl of purified SSA (1 mg/ml), or OVA (1 mg/ml), or vehicle control (PBS) was intratracheally (i.t.) instilled. AHR, mucus production, and eosinophilia were measured on day 17 (Fig. 1*A*). To investigate whether oral administration of SSA-lupin could protect against the development of experimental asthma, mice were i.g. administered 250 μl of SSA-lupin or lupin seed suspension twice per week for 4 wk. One week following i.g. challenge, mice received an i.p. injection of 50 μg of SSA dissolved in PBS with alum (1 mg/ml). Following i.p. immunization, mice received i.t. administration of SSA (25 μg/25 μl PBS) on days 49 and 51 (Fig. 1*C*). Airways hyperresponsiveness to methacholine was assessed in conscious, unrestrained mice by barometric plethysmography, using apparatus and software supplied by Buxco (Troy, NY). This system yields a dimensionless parameter known as enhanced pause (Penh), reflecting changes in wave form of the pressure signal from the plethysmography chamber combined with a timing comparison of early and late expiration, which can be used to empirically monitor airway function. Measurement was performed, as previously described (36). Lung tissue representing the central (bronchi-bronchiole) and peripheral (alveoli) airways was fixed, processed, and stained with Alcian blue-periodic acid-Schiff for enumeration of mucin-secreting cells or Charbol's chromotrope-hematoxylin for identification of eosinophils, as previously described (36). The number of mucus-staining cells and eosinophils in the central bronchi-bronchiole area was identified by morphological criteria and quantified, as previously described (36).

Determination of SSA-specific IgE, IgG1, and IgG2a in serum

Serum was analyzed at the completion of the experimental asthma protocol described in Fig. 1*A* (day 17). Ab titers were determined, as previously described (37). In brief, whole blood samples from lupin- and SSA-lupin-fed mice were taken and microcentrifuged at 13,000 rpm for 5 min at room temperature. Serum was extracted and stored at −70°C until analysis. SSA-specific IgG1 and IgG2a levels were determined using an ELISA. Nunc Maxisorb ELISA 96-well plates were coated with SSA (100 μg/ml) and incubated overnight at 4°C. For measurements determining SSA-specific IgE Ab titers, Nunc Maxisorb ELISA 96-well plates were coated with anti-mouse IgE (rat anti-mouse IgE; 1.6 μg/ml; BD PharMingen, San Diego, CA). The plates were then washed with 0.05% Tween 20 in PBS, blocked with 10% FCS in PBS, and incubated at 37°C for 2 h. Following blocking, plates were washed with 0.05% Tween 20 in PBS, and 100 μl of serum samples was added. Serum sample was diluted 1/10 in 10% FCS. After a 2-h incubation at 37°C, plates were washed with 0.05% Tween 20 in PBS and incubated for 1 h at 37°C with 100 μl of biotin-conjugated anti-mouse IgG1 (goat anti-mouse IgG1 biotinylated; 0.5 μg/ml; Southern Biotechnology Associates, Birmingham, AL) or IgG2a (goat anti-mouse IgG1 biotinylated; 0.5 μg/ml; Southern Biotechnology Associates) or biotin-conjugated SSA (200 μg/ml). Plates were again washed with 0.05% Tween 20 in PBS, and serum Ab levels were detected using streptavidin HRP detection system (100 μl; 2 μg/ml). The plates were then developed with 100 μl of BD PharMingen substrate kit, and the reaction was stopped with 2 N H_2SO_4. OD were read at 450–570 nm using a 96-well MR-600 microplate reader (Molecular Devices, Sunnyvale, CA). Data are expressed as mean OD at the stated serum dilution.

Delayed-type hypersensitivity (DTH) responses

The experimental regime used to examine systemic sensitization by oral administration of SSA-lupin is described in Fig. 1*B*. In brief, 1 wk following the final i.g. dosage of lupin and SSA-lupin seed meal, mice were s.c. injected into the footpad with 50 μl of SSA or OVA at 1 mg/ml. As a positive control ((+) control (24 h)), mice were i.p. injected with SSA/CFA (50 μg SSA/1 mg CFA), and 14 days later subsequently received SSA (50 μg/50 μl PBS) s.c. in the hind footpad, and DTH responses were measured 24 h later. DTH responses were assessed by measuring the specific increase in footpad thickness using a digmatic caliper (Mitutoyo, Kawasaki, Japan) 24 h following challenge.

Histological analysis of eosinophils in the lung tissue

The lung of mice was fixed in 4% paraformaldehyde, embedded in paraffin, cut into 5-μm sections, fixed to slides, and immunostained with antiserum against mouse eosinophil major basic protein (anti-MBP), as described (38, 39). In brief, endogenous peroxidase in the tissues was quenched with 0.3% hydrogen peroxide in methanol, followed by nonspecific protein blocking with normal goat serum. Tissue sections were then incubated with rabbit anti-MBP (1:16,000) overnight at 4°C, followed by 1/200 dilution of biotinylated goat anti-rabbit IgG secondary Ab and avidin-peroxidase complex (Vector Laboratories, Burlingame, CA) for 30 min each. These slides were further developed with nickel diaminobenzidine-cobalt chloride solution to form a black precipitate, and counterstained with nuclear Fast Red. Replacing the primary Ab with normal rabbit serum ablated the immunostaining, as reported (38).

Ag-specific $CD4^+$ T cell response

Peribronchial lymph node (PBLN) cells were subjected to SSA or αCD3/αCD28 stimulation, as previously described (40). In brief, 5×10^5 cells were cultured with SSA (50 μg/ml) or αCD3 (5 μg/ml)/αCD28 (1 μg/ml) for 96 h. IL-4, IL-5, IL-10, and IFN-γ levels were determined in supernatants from stimulated PBLN homogenates by using the OptEIA mouse IL-4, IL-5, IL-10, and IFN-γ kits (BD PharMingen). The IL-13 levels were measured by using rat anti-mouse IL-13 mAb (R&D Systems, Minneapolis, MN; clone 38213.11) and biotinylated rat anti-mouse IL-13 mAb (R&D Systems).

Purification of $CD4^+CD25^+$, $CD4^+CD25^-$, $CD4^+CD45^{high}$ T cells, and $CD4^+CD45^{low}$ T cell subpopulations from SSA-lupin-fed mice

BALB/c mice were fed SSA-lupin, as described above. Mice were exsanguinated, and spleens and mesenteric lymph nodes were removed and prepared into single cell suspensions, as previously described (41). To prepare $CD4^+CD25^+$ T cell- and $CD4^+CD25^-$ T cell-purified populations, splenocytes were incubated with PE-conjugated anti-CD25 (clone PC61; BD PharMingen) (10 μg/10^8 cells) and FITC-conjugated anti-CD4 (clone

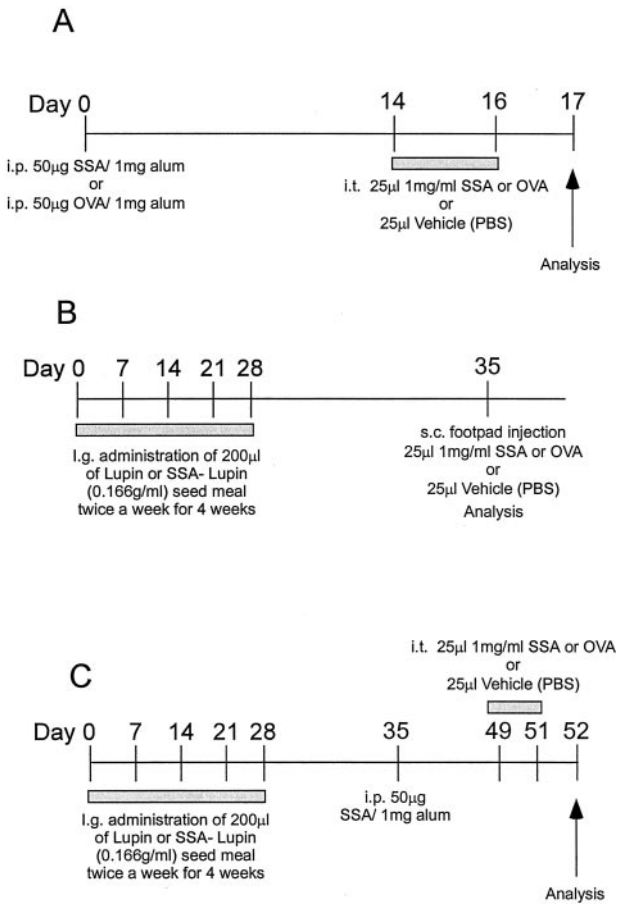

FIGURE 1. Experimental regimes. *A*, Murine model of experimental asthma. Mice were immunized by an i.p. injection of 200 μl containing 50 μg of SSA dissolved in PBS with alum (1 mg/ml). After i.p. immunization, mice received i.t. administration of SSA or OVA (50 mg/25 μl PBS) on days 14 and 16. *B*, Lupin and SSA-lupin induced systemic sensitization and DTH responses. Mice were i.g. administered 250 μl of lupin or SSA-lupin seed meal, twice per week for 4 consecutive wk. One week following the last i.g. challenge, mice received s.c. administration of SSA (50 μg/50 μl PBS) in the hind footpad, and DTH responses were measured. As a positive control ((+) control (24 h)), mice were i.p. injected with SSA/CFA (50 μg SSA/1 mg CFA), and 14 days later subsequently received SSA (50 μg/50 μl PBS) s.c. in the hind footpad, and DTH responses were measured 24 h later. Hypersensitivity responses were determined, as described in *Materials and Methods*. *C*, Lupin and SSA-lupin mediated suppression of experimental asthma. Mice were i.g. administered 250 μl of lupin or SSA-lupin seed meal twice per week for 4 consecutive wk. One week following i.g. challenge, mice received an i.p. injection of 50 μg of SSA dissolved in PBS with alum (1 mg/ml). After immunization, mice received i.t. administration of SSA (25 μg/25 μl PBS) on days 49 and 51. Analysis of AAD was performed as described in *Materials and Methods*.

L3T4; BD PharMingen) (10 μg/10^8 cells) in PBS/1% FCS on ice for 30 min and washed twice in PBS/1% FCS. To prepare $CD4^+CD45^{high}$ T cell- and $CD4^+CD45^{low}$ T cell-purified populations, mesenteric lymph node cells were incubated with FITC-conjugated anti-CD45RB (clone 16A; BD PharMingen) (10 μg/10^8 cells) and PE-conjugated anti-CD4 (clone L3T4; BD PharMingen) (10 μg/10^8 cells) in PBS/1% FCS on ice for 30 min and washed twice in PBS/1% FCS. Cells were sorted by flow cytometry on a FACSVantage SE cell sorter (BD Biosciences, San Jose, CA). The purity of $CD4^+$ T cell subpopulations was >95% on reanalysis.

Cell transfer system for $CD4^+$ T cell suppression of AAD

Either purified $CD4^+CD25^+$ T cells or $CD4^+CD25^-$ T cells (2×10^6 cells/mouse) or $CD4^+CD45^{high}$ T cells and $CD4^+CD45^{low}$ T cells (1×10^6 cells/mouse) from SSA-lupin-fed mice were injected i.v. to naive BALB/c mice on day 0. On day 0, the mice also received an i.p. injection of 50 μg of SSA in alum (1 mg/ml) dissolved in PBS (final volume 200 μl). On days 14 and 16, the mice were anesthetized with an i.v. injection of 100 μl Saffan solution (1/4 diluted in water). Mice were intubated with a 22-gauge catheter needle, and 25 μl of purified SSA (1 mg/ml PBS), OVA (1 mg/ml), or vehicle control (PBS) was i.t. instilled. AHR, mucus production, and eosinophilia were measured on day 17 (Fig. 1*A*).

Statistical analysis

The significance of differences between experimental groups was analyzed using Student's unpaired *t* test. Values are reported as the mean + SEM. Differences in means were considered significant if $p < 0.05$.

Results

Characterization of AAD in SSA-sensitized and challenged BALB/c mice

Initially, we were interested in assessing whether purified SSA could promote experimental asthma and to compare inflammation with the established disease model induced with OVA (42, 43). Mice were i.p. sensitized with SSA and alum, and subsequently i.t. challenged with either SSA or vehicle control PBS to induce experimental asthma (Fig. 1*A*). Vehicle control (PBS) i.t. challenge of SSA-sensitized mice did not induce mucus hypersecretion (Fig. 2*A*), eosinophil accumulation in the pulmonary compartment (Fig. 2*B*), nor enhanced airways hyperresponsiveness (Fig. 2*C*). In contrast, i.t. SSA challenge of SSA-primed mice induced a significant increase in mucus secretion, pulmonary eosinophils, and airways hyperresponsiveness (Fig. 2, *A–C*). Interestingly, the degree of mucus hyperscretion, pulmonary eosinophilic inflammation, and

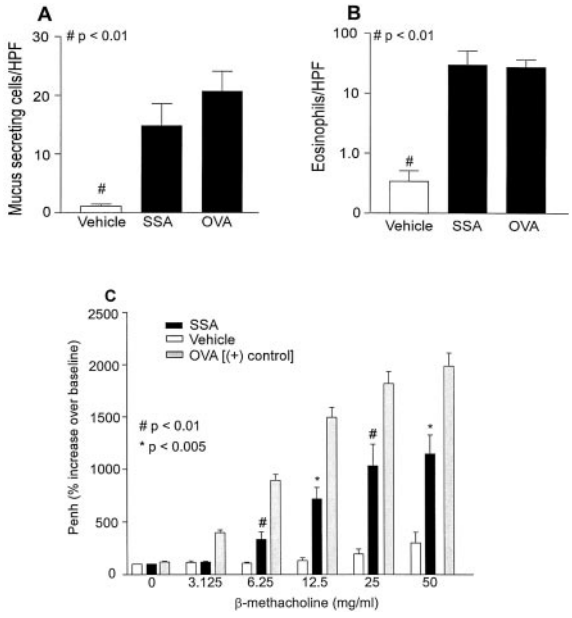

FIGURE 2. Characterization of asthma indices in SSA-sensitized and challenged mice. *A*, Mucus-secreting cells; *B*, eosinophils/HPF in the lung; and *C*, AHR in SSA- or OVA-sensitized and challenged mice. Experimental asthma was induced, as described in Fig. 1. *A* and *B*, Mucus-secreting cells and eosinophils in lung tissue were determined, as described in *Materials and Methods*. Data are the means ± SEM from $n = 3$–5 mice per group and representative of duplicate experiments. Statistical significance of differences ($p < 0.05$, $p < 0.01$, and $p < 0.005$) was determined using Student's unpaired *t* test. *C*, AHR in response to methacholine was determined as the percentage increase in Penh, as described in *Materials and Methods*, and was assessed 24 h following i.t. challenge. Data shown represent the mean Penh (% increase over baseline) ± SEM (where $n = 8$) and are representative of triplicate experiments. Statistical significance of differences ($p < 0.005$ and $p < 0.01$) was determined using Student's unpaired *t* test.

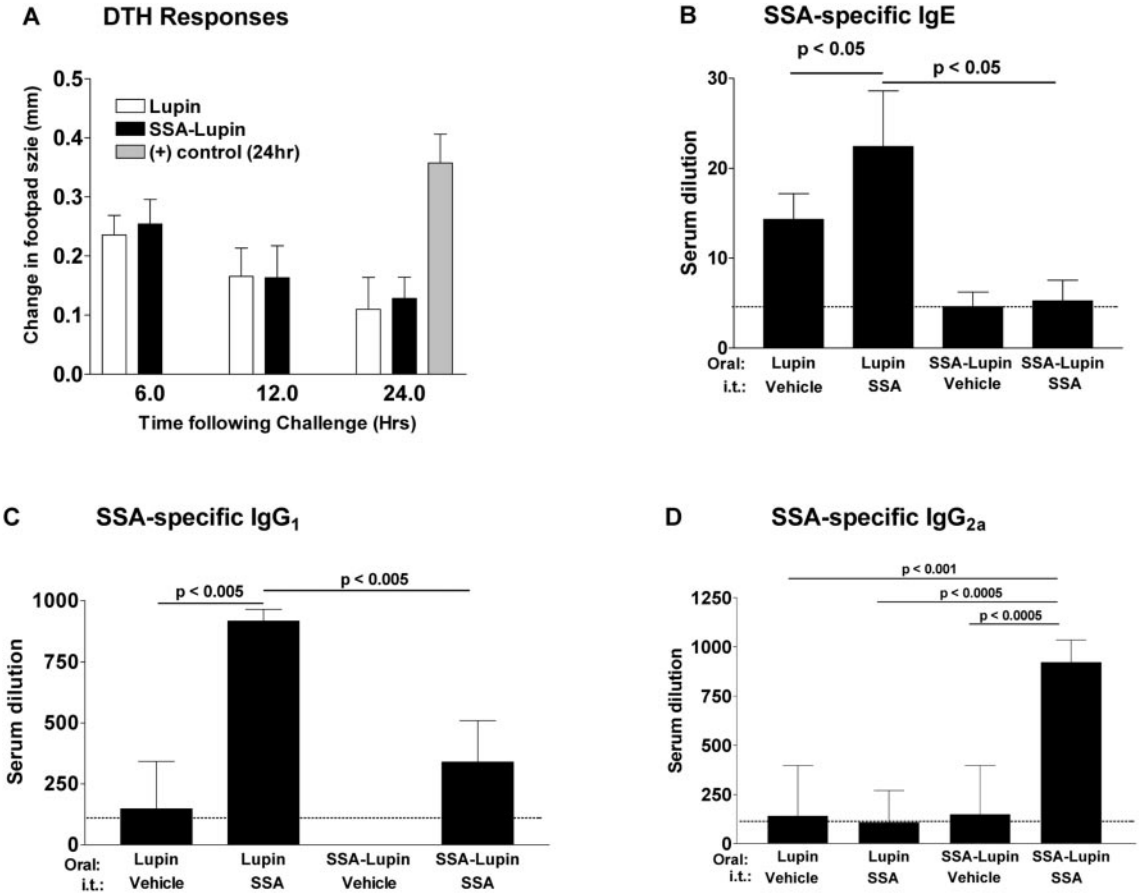

FIGURE 3. SSA-specific IgG1 and IgG2a levels in serum and DTH responses of SSA- or vehicle-challenged mice fed lupin or SSA-lupin seed meal. *A*, DTH responses; *B*, serum SSA-specific IgG1; *C*, IgE; and *D*, IgG2a levels were determined in SSA- or vehicle-challenged mice that were fed lupin and SSA-lupin seed meal. *A*, Hypersensitivity responses were determined in lupin- and SSA-lupin-fed mice, as described in *Materials and Methods* and Fig. 1*B*. As a positive control ((+) control (24 h)), mice were i.p. injected with SSA/CFA (50 μg SSA/1 mg CFA). On day 14, mice were subsequently s.c. administered SSA (25 μg/μl PBS) in the hind footpad, and DTH responses were measured 24 h later. Data are representative of the mean increase in footpad width (mm) $\pm$ SEM from $n = 4$–5 mice per group from duplicate experiments. Statistical significance of differences ($p < 0.05$) was determined using Student's unpaired t test. *B* and *D*, SSA-specific IgE, IgG1, and IgG2a titers were determined using ELISA, as previously described in *Materials and Methods*. Data represent mean serum dilution (required to obtain an OD 0.165) $\pm$ SEM from $n = 4$–6 mice per group. Statistical significance of differences ($p < 0.05$) was determined using Student's t test.

AHR observed in SSA-challenged, SSA-primed mice was comparable to that observed in mice primed and challenged with the established disease model Ag OVA (Fig. 2, *A–C*). Collectively, these experiments show that purified SSA can induce experimental asthma and promote an inflammatory response comparable to that of the established disease model Ag OVA.

Recently, there has been significant concern that a GM plant expressing a foreign protein that is a known allergen may predispose to hypersensitivity responses following subsequent exposure to the transgenically expressed protein (44–46). Therefore, we established whether oral administration of our vaccine candidate SSA-lupin predisposes to systemic SSA-specific DTH responses. Mice were given lupin or SSA-lupin twice per week for 4 wk and then subsequently challenged s.c. in the hind footpad with SSA and DTH responses measured (Fig. 1*B*). Oral consumption of SSA-lupin did not predispose the mice to SSA-hypersensitivity reactions (Fig. 3*A*). No significant difference in footpad size was observed between SSA-challenged lupin- and SSA-lupin-fed mice. As a positive control for DTH responses, mice were sensitized i.p. with (50 μg) OVA in CFA, and subsequently s.c. challenged in the hind footpad with OVA (50 μg/50 μl) (Fig. 3*A*; 24-h time point shown). Collectively, these studies demonstrate that consumption of SSA-lupin does not systemically sensitize animals and predispose to an SSA-specific DTH response.

To elucidate whether oral consumption of a GM plant expressing a potential allergen could suppress experimental allergy, mice were orally administered lupin or SSA-lupin meal and then subsequently primed i.p. and i.t. challenged with SSA (Fig. 1*C*). To determine the type of immune response elicited against SSA following oral administration of SSA-lupin, we examined serum SSA-specific IgE, IgG1, and IgG2a Ab titers. Intratracheal challenge of lupin-fed mice with SSA promoted a significant SSA-specific IgE and IgG1 response, but no detectable Ag-specific IgG2a. In contrast, i.t. challenge of SSA-lupin-fed mice with SSA induced an SSA-specific IgG2a, but no IgE or IgG1 response (Fig. 3, *B–D*). Collectively, these data suggest that oral administration of SSA promoted an Ag-specific Th1-type as opposed to a Th2-type immune response. To further examine the ability of a GM plant expressing a potential allergen to attenuate experimental asthma, mice were orally administered with lupin or SSA-lupin and subsequently primed i.p. and i.t. challenged with SSA or vehicle, and the asthma indices were determined (mucus hypersecretion, pulmonary eosinophilia, and AHR). SSA sensitization and challenge of lupin-fed mice promoted experimental allergy, as characterized

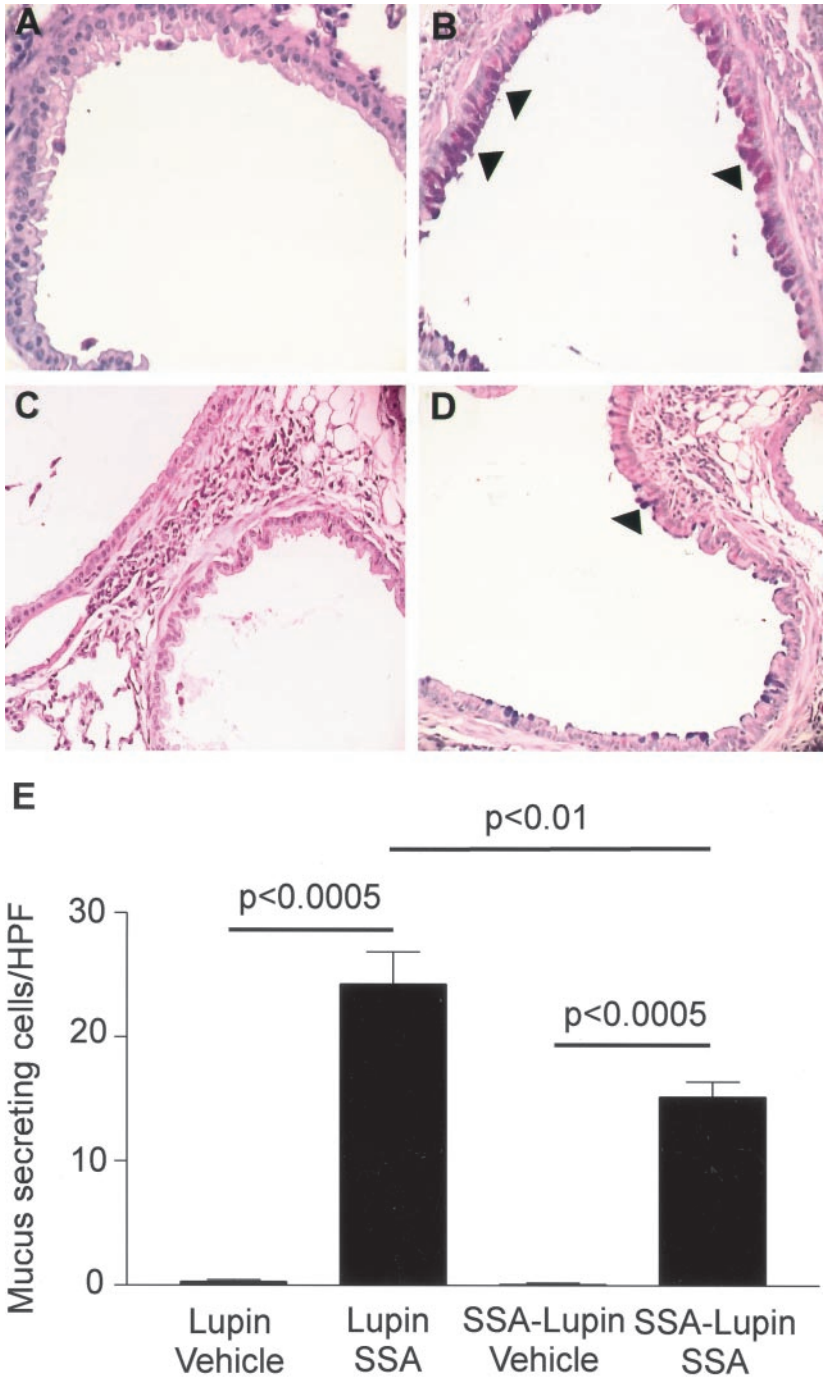

FIGURE 4. Characterization of pulmonary mucus hypersecretion in SSA-primed and i.t. challenged mice following i.g. administered lupin or SSA-lupin seed meal. *A–E*, Representative photomicrographs of lung sections from *A*, lupin-fed mice primed with SSA and i.t. challenged with vehicle; *B*, lupin-fed mice primed and i.t. challenged with SSA; *C*, SSA-lupin-fed mice primed with SSA and i.t. challenged with vehicle; and *D*, SSA-lupin-fed mice primed and i.t. challenged with SSA. *E*, Quantification of mucus-secreting cells in lung tissue from SSA- or vehicle-challenged mice i.g. administered with lupin or SSA-lupin seed meal. Mucus-secreting cell numbers in lung tissue were determined in SSA- or vehicle-challenged mice i.g. administered with lupin or SSA-lupin seed meal, as described in *Materials and Methods*. Data are the mean mucus-secreting cell numbers/HPF ± SEM from $n = 3$–5 mice per group from duplicate experiments. Statistical significance of differences ($p < 0.05$ and $p < 0.0005$) was determined using Student's unpaired t test. Mice were exposed to the experimental regime described in Fig. 1*C*. Full arrow depicts mucus-secreting cells.

by mucus hypersecretion, pulmonary eosinophilia, and AHR (Figs. 4, 5, and 6). Intratracheal challenge of lupin-fed mice with SSA promoted eosinophil recruitment into the pulmonary compartment that was predominantly localized in the subepithelial region (Fig. 5, *B* and *C*). Vehicle challenge of SSA-lupin-fed mice did not induce any significant difference in the experimental asthma indices as compared with lupin-fed mice. Notably, experimental allergy as assessed by mucus, eosinophil levels in the airways, and AHR was suppressed in SSA-challenged mice fed SSA-lupin as compared with SSA-challenged mice fed lupin (Figs. 4, 5, and 6). Although minor inflammation was observed in the pulmonary compartment of SSA-challenged mice fed SSA-lupin, the cellular infiltrate was predominantly characterized by mononuclear cells, and not eosinophils (Fig. 5*E*). Collectively, these data indicate that oral administration of a GM plant expressing a potential allergen can attenuate experimental asthma caused by that protein.

To determine whether the suppression of experimental allergy in SSA-lupin-fed mice was associated with a reduction in $CD4^+$ T cell-derived Th2 cytokines, cytokine responses by PBLN T cells from SSA- or vehicle-challenged lupin- and SSA-lupin-fed mice were determined (Fig. 7, *A–D*). Cytokine profiles from PBLN T cells following polyclonal activation with αCD3/αCD28 were similar, indicating that maximal proliferation rates and levels of cytokine production were comparable between groups (Fig. 7, *A–D*). SSA stimulation of PBLN T cells from SSA-challenged lupin-fed mice induced the production of the Th2 cytokines IL-5 and IL-13, but no detectable IFN-γ (Fig. 7, *A–C*). Similarly, addition of SSA to PBLN T cells of SSA-challenged SSA-lupin-fed

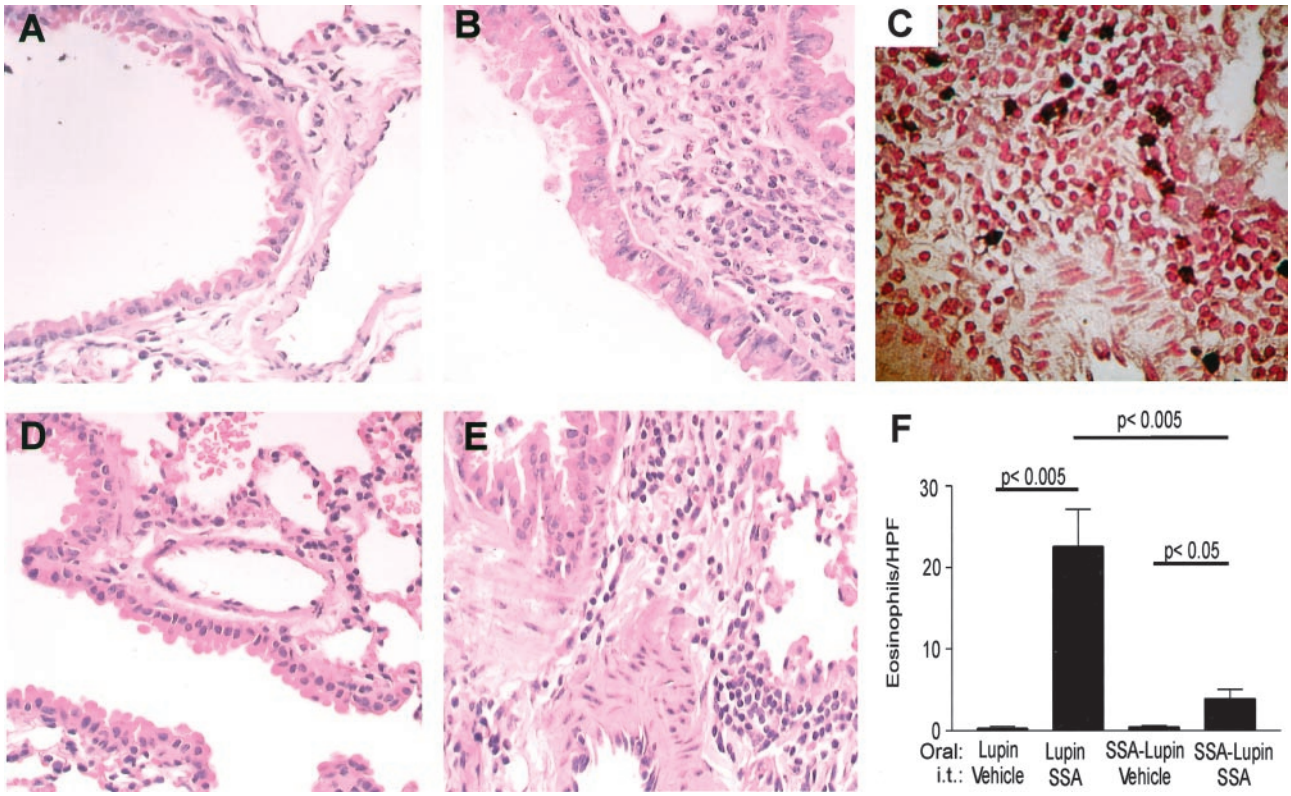

FIGURE 5. Characterization of pulmonary eosinophilic infiltration in SSA-primed and i.t. challenged mice following i.g. administered lupin or SSA-lupin seed meal. *A–E*, Representative photomicrographs of lung sections from *A*, lupin-fed mice primed with SSA and i.t. challenged with vehicle; *B*, lupin-fed mice primed and i.t. challenged with SSA; *C*, high magnification of anti-MBP-stained lung tissue of lupin-fed mice primed and i.t. challenged with SSA; *D*, SSA-lupin-fed mice primed with SSA and i.t. challenged with vehicle; and *E*, SSA-lupin-fed mice primed and i.t. challenged with SSA. *F*, Quantification of eosinophil numbers in lung tissue from SSA- or vehicle-challenged mice i.g. administered with lupin or SSA-lupin seed meal. Eosinophil numbers in lung tissue were determined in SSA- or vehicle-challenged mice i.g. administered with lupin or SSA-lupin seed meal, as described in *Materials and Methods*. Data are the mean eosinophils/HPF ± SEM from $n = 3–5$ mice per group from triplicate experiments. Statistical significance of differences ($p < 0.05$ and $p < 0.0005$) was determined using Student's unpaired *t* test. Full arrow depicts eosinophils.

mice also promoted the production of the Th2 cytokines IL-5 and IL-13 (Fig. 7, *A* and *C*). The levels of IL-5 and IL-13 were comparable to those observed in PBLN T cell cultures from SSA-challenged lupin-fed mice (Fig. 7, *A* and *C*). However, in contrast to lupin-fed mice, SSA stimulation of T cells from SSA-lupin-fed mice also induced the production of Th1 cytokine IFN-γ (Fig. 7*B*). Collectively, these data indicate that oral administration of a GM plant expressing an allergen can promote the expansion of a Th1-type protective immune response in addition to the Ag-specific Th2-type response.

To determine whether the suppression of experimental allergy in SSA-lupin-fed mice was associated with the production of $CD4^+$ T cell-derived suppressor cytokines, IL-10 levels in PBLN T cell cultures from SSA- or vehicle-challenged lupin and SSA-lupin mice were determined (Fig. 7*D*). SSA or polyclonal stimulation of PBLN T cells from SSA-challenged lupin-fed mice did not promote the production of IL-10. In contrast to lupin-fed mice, SSA or polyclonal stimulation of PBLN T cells from SSA-lupin-fed mice induced the production of IL-10 (Fig. 7*D*). Interestingly, we did not observe any significant change in IL-10 levels in T cell cultures from SSA-challenged lupin-fed mice following polyclonal T cell activation (Fig. 7*D*).

To identify the contribution of IL-10 in lupin-SSA-mediated suppression of AAD, we adoptively transferred purified mesenteric lymph node-derived $CD4^+$ T cells from SSA-lupin-fed WT and IL-10-deficient mice into naive mice and attempted to induce AAD. Sensitization and i.t. challenge of naive mice with SSA receiving WT or IL-10-deficient $CD4^+$ T cells from lupin-fed mice induced experimental allergy (eosinophils/high power field (HPF), 16.1 ± 7.4 vs 20.72 ± 10.4; mucus-secreting cells/HPF, 14.29 ± 7.2 vs 24.5 ± 4.3; Penh (% increase over baseline) at 25 mg/ml methacholine, 1157.72 ± 418.80 vs 1156.965 ± 346.9; mean ± SD, $n = 4–6$ mice; WT $CD4^+$ T cell recipient mice compared with IL-10-deficient T cell recipient mice, respectively), with mucus, eosinophil levels in the airways, and AHR being significantly enhanced in these mice as compared with vehicle-challenged group (eosinophils/HPF, 1.30 ± 0.15; mucus-secreting cells/HPF, 0.34 ± 0.16; Penh (% increase over baseline) at 25 mg/ml methacholine, 301.945 ± 101.07; mean ± SD, $n = 4–6$ mice; WT $CD4^+$ T cell recipient mice; $p < 0.05$). Interestingly, sensitization and i.t. challenge of naive mice with SSA receiving WT or IL-10-deficient $CD4^+$ T cells from SSA-lupin-fed mice significantly suppressed the asthma phenotype as compared with SSA-sensitized and i.t. challenged naive mice receiving IL-10-deficient $CD4^+$ T cells from lupin-fed mice ($p < 0.05$) (eosinophils/HPF, 4.9 ± 2.6 vs 8.98 ± 4.9; mucus-secreting cells/HPF, 11.1 ± 3.2 vs 9.9 ± 3.7; Penh (% increase over baseline) 25 mg/ml methacholine, 642.2 ± 158.3 vs 513.3 ± 275.9; mean ± SD, $n = 4–6$ mice; WT $CD4^+$ T cell recipient mice compared with IL-10-deficient T cell recipient mice, respectively). Collectively, these studies suggested that IL-10 is not critical in GM plant-mediated suppression of experimental asthma.

IFN-γ is a Th1-type cytokine that down-regulates Th2-type immune responses both in vitro and in vivo (47, 48). Interestingly, we

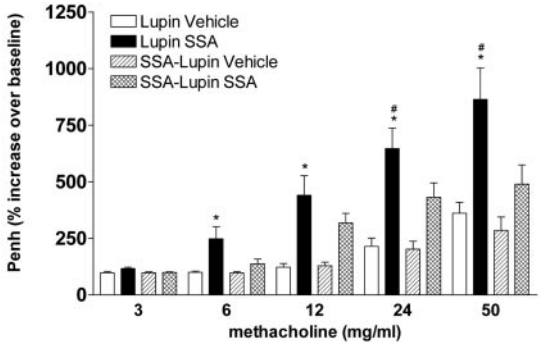

FIGURE 6. Airway reactivity to β-methacholine in SSA- or vehicle-challenged mice following i.g. administered lupin or SSA-lupin seed meal. AHR in response to β-methacholine was determined in SSA-sensitized and SSA- or vehicle-challenged mice that were i.g. administered with lupin or SSA-lupin seed meal. Mice were exposed to the experimental regime described in Fig. 1*C*. Data shown represent the mean Penh (% increase over baseline) ± SEM (where $n = 8–10$) from duplicate experiments. Cumulative dose-response curve to methacholine (1, 3, 6, 12, 24, and 50 mg/ml) challenge was measured by barometric plethysmography, and the data represent the percentage increase in Penh over baseline reactivity. Statistical significance ($p < 0.05$) of differences was determined using Student's unpaired *t* test.

observed elevated levels of IFN-γ in PBLN-derived T cell culture supernatants from SSA-lupin-fed, but not in lupin-fed SSA-sensitized and challenged WT mice. Consistent with this finding, we observed elevated levels of IFN-γ-regulated Ag-specific IgG2a Abs in the serum of SSA-lupin-fed SSA-sensitized and challenged WT mice, suggesting that IFN-γ may play a role in lupin-SSA-mediated suppression of AAD (Fig. 3*D*). To examine the role of IFN-γ in GM plant-induced suppression of AAD, we fed IFN-γ-deficient and WT mice with lupin or SSA-lupin. Sensitization and i.t. challenge of lupin-fed WT mice with SSA induced experimental allergy, with mucus and eosinophil levels in the airways and AHR, whereas treatment of SSA-lupin-fed WT mice suppressed AAD (Fig. 8, *A–C*). Interestingly, in the absence of IFN-γ, the protective effect of SSA-lupin on the induction of AAD was lost. The level of experimental asthma (mucus production, pulmonary eosinophilia, and AHR) in SSA-lupin-fed IFN-γ-deficient mice was comparable to that observed in lupin-fed IFN-γ-deficient mice (Fig. 8, *A–C*). Collectively, these studies suggest a critical role for IFN-γ in GM plant-mediated suppression of experimental asthma.

$CD4^+CD45RB^{low}$ regulatory T cells have been shown to possess regulatory T cell capabilities inhibiting the development of inflammatory disease processes, including inflammatory bowel disease, graft tissue rejection, and allergic pulmonary inflammation (12, 49, 50). To determine whether $CD4^+CD45RB^{low}$ regulatory T cells mediated GM plant suppression of AAD, we adoptively transferred purified $CD4^+CD45RB^{high}$ or $CD4^+CD45RB^{low}$ T cells from draining mesenteric lymph nodes of SSA-lupin-fed mice to naive syngeneic mice. $CD4^+CD45RB^{high}$ or $CD4^+CD45RB^{low}$ T cells were purified from draining mesenteric lymph nodes of SSA-lupin-fed mice (Fig. 9, *A–D*). The purity of the enriched $CD4^+CD45RB^{high}$ or $CD4^+CD45RB^{low}$ T cells was greater than 95% (Fig. 9*D*). Intratracheal administration of SSA to SSA-sensitized BALB/c mice receiving purified $CD4^+CD45RB^{high}$ T cells induced the asthma phenotype (eosinophilic infiltration, mucus hypersecretion, and airways hyperresponsiveness) (Fig. 9, *E–G*). Interestingly, the asthma phenotype was significantly attenuated in SSA i.t. challenged and sensitized BALB/c mice pretreated with purified $CD4^+CD45RB^{low}$ regulatory T cells (Fig. 9, *E–G*). The level of AAD in $CD4^+CD45RB^{low}$ T cell-pretreated mice was equivalent to that observed in vehicle i.t. challenged $CD4^+CD45RB^{low}$ T cell-pretreated mice.

Discussion

SSA is a member of the 2S methionine-rich protein family (51). Interestingly, proteins in the 2S family from rapeseed, castor

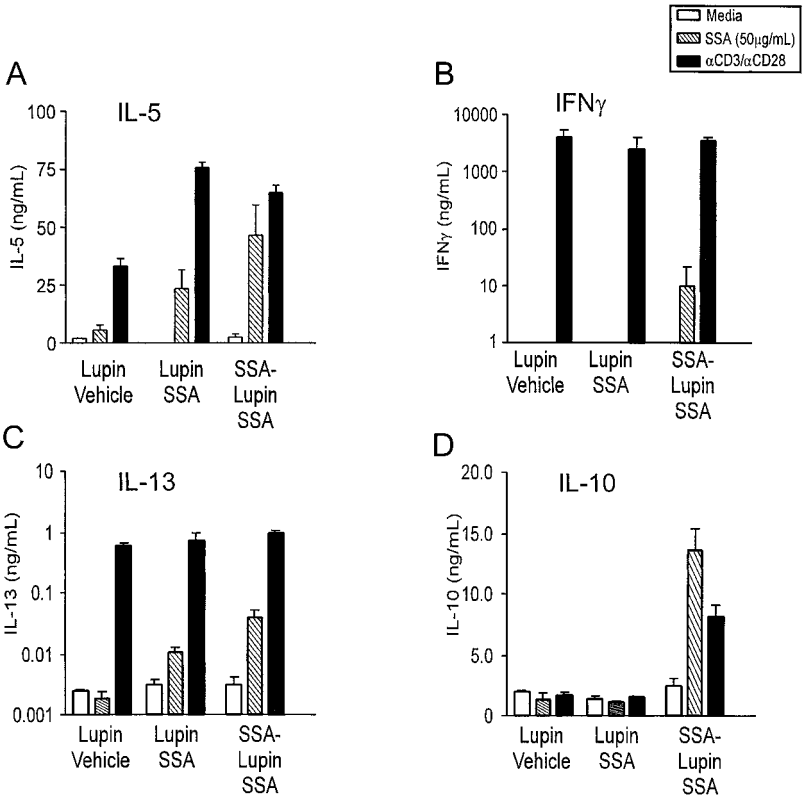

FIGURE 7. Characterization of PBLN T cell-derived cytokine production from vehicle- or SSA-challenged mice following i.g. administered lupin and SSA-lupin seed meal. IL-5 (*A*), IL-13 (*B*), IFN-γ (*C*), and IL-10 (*D*) levels in supernatants from αCD3/αCD28 or SSA-stimulated PBLN cells from SSA- or vehicle-challenged mice i.g. administered with lupin or SSA-lupin seed meal. Mice were exposed to the experimental regime described in Fig. 1*C*. Twenty-four hours following the last challenge, the mice were sacrificed, and PBLNs were excised and cultured, as described in *Materials and Methods*. Data expressed as mean cytokine concentration (nanograms per milliliter) ± SEM level of cytokine from $n = 4–5$ mice per group from triplicate cultures.

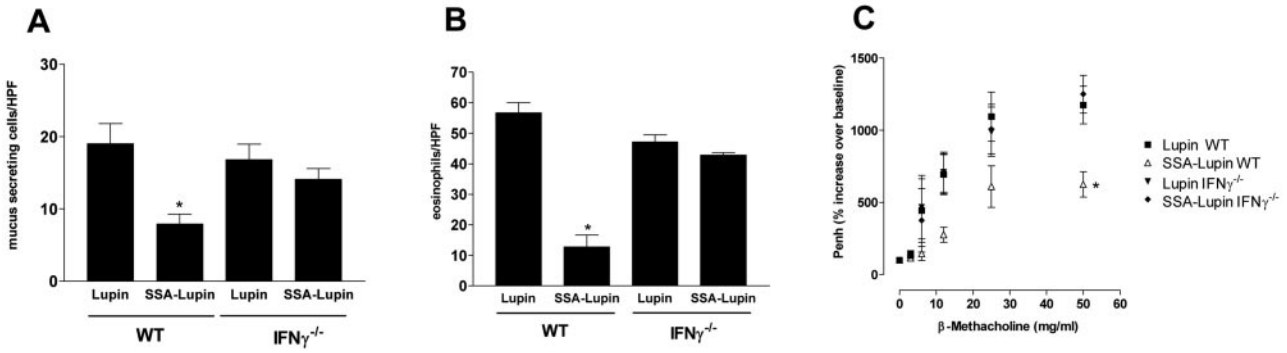

FIGURE 8. Characterization of pulmonary eosinophilic infiltration, mucus hypersecretion, and airways reactivity in SSA-primed and i.t. challenged WT and IFN-$\gamma^{-/-}$ mice following i.g. administered lupin or SSA-lupin seed meal. *A*, Mucus-secreting cells/HPF; *B*, eosinophil numbers/HPF in the lung of lupin- and SSA-lupin-fed WT and IFN-$\gamma^{-/-}$ mice primed and i.t. challenged with SSA. *C*, AHR response to methacholine in lupin- and SSA-lupin-fed WT and IFN-$\gamma^{-/-}$ mice primed and i.t. challenged with SSA. Mucus-secreting cell numbers in lung tissue were determined, as described in *Materials and Methods*. Data are the mean mucus-secreting cell numbers/HPF ± SEM from $n = 7$–10 mice per group. *B*, Eosinophil numbers in lung tissue were determined, as described in *Materials and Methods*. Seven similar fields were counted per lung. Data are the mean eosinophil numbers/HPF ± SEM from $n = 7$–10 mice per group from duplicate experiments. *C*, Data shown represent the mean Penh (% increase over baseline) ± SEM (where $n = 8$–10 mice per group). Cumulative dose-response curve to methacholine (1, 3, 6, 12, 24, and 50 mg/ml) challenge was measured by barometric plethysmography, and the data represent the percentage increase in Penh over baseline reactivity. Statistical significance ($p < 0.05$) of differences was determined using Student's unpaired *t* test. *, $p < 0.05$ as compared with SSA-lupin IFN-$\gamma^{-/-}$.

beans, cottonseed, walnuts, and Brazil nuts have been associated with atopy (52–56). Recently, it has been proposed that SSA may contain IgE-binding epitopes and could be the primary allergenic protein in sunflower seed hypersensitivity (33, 34). Using a GM plant expressing the allergen, SSA, and an experimental model of AAD, we were interested in determining whether oral consumption of a GM plant could protect against any SSA-mediated allergic disease. In the present study, we show for the first time that: 1) oral consumption of SSA-lupin promoted a SSA-specific Th1-type Ab response (IgG2a); 2) oral consumption of SSA-lupin does not predispose to DTH responses; 3) oral consumption of SSA-lupin can suppress the asthma phenotype (mucus hypersecretion, eosinophil accumulation, and AHR); 4) lupin-SSA-mediated suppression of AAD was associated with Ag-specific $CD4^+$ T cell-derived IFN-γ and IL-10 production; 5) lupin-SSA-mediated suppression of AAD was dependent on IFN-γ; and 6) $CD4^+CD45RB^{low}$ T cells induced by oral consumption of GM plant can mediate suppression of AAD.

Three classes of Ag-specific suppressive T cell populations, Th3 ($TGF\beta^+$) T cells, T regulatory (Tr1 IL-4/IL-10^+) cells, and $CD4^+CD25^+$ regulatory T cells, have been identified and are thought to play a role in oral tolerance and immune suppression (15, 21, 57). In the present study, we demonstrate that $CD4^+CD45RB^{low}$ T cells play a central role in GM plant-mediated suppression of AAD. In support of this observation, recent studies have shown that $CD4^+CD45RB^{low}$ T cells can inhibit the development of immunopathological disease processes, including tissue graft rejection, *M. vaccae*-induced suppression of AAD, and inflammatory bowel disease (49, 50). Interestingly, inhibition of AAD was associated with Ag-specific IgG2a Ab response and $CD4^+$ T cell-derived IFN-γ production. By using IFN-γ-deficient mice, we show that GM plant-mediated suppression of AAD was highly dependent on IFN-γ. In vitro and in vivo studies have demonstrated a role for IFN-γ in the inhibition of $CD4^+$ Th2-type-mediated disease processes such as asthma (8, 58–60). IFN-γ has been shown in vitro to suppress the differentiation and expansion of $CD4^+$ Th2-type T cells and can also down-regulate Th2-type cytokine production (47, 48). Furthermore, administration of recombinant IFN-γ or $CD4^+$ IFN-γ-expressing T cells or mucosal IFN-γ gene transfer inhibits Th2-mediated AAD (8, 59, 60).

Previous investigations using experimental models of inflammatory bowel disease, graft rejection, and *M. vaccae*-mediated suppression of asthma have demonstrated that the specific inhibitory properties of $CD4^+CD45RB^{low}$ regulatory T cells are mediated via IL-10 and $TGF\beta$ (12, 49, 50). Furthermore, experimental models of allergic disease have demonstrated that IL-10 can act as a natural suppressor of Th2-type cytokine production and inflammation (61–63). IL-10 is thought to act via a number of regulatory mechanisms, including down-regulation of mast cell function, suppression of IgE, and eosinophil-sensitive chemokine production and reduced eosinophil survival (64, 65). Although we demonstrate that suppression of experimental allergy by GM plants is associated with the development of a pronounced Ag-specific $CD4^+$ T cell-derived IL-10 response, we were unable to demonstrate any significant contribution of IL-10 in GM plant-mediated suppression of AAD. We observed no attenuation in suppression of AAD in IL-10-deficient mice following consumption of lupin-SSA- or in SSA-sensitized i.t. challenged mice receiving IL-10-deficient $CD4^+$ T cells from lupin-SSA-fed mice. Similarly, we did not observe any significant contribution by $CD4^+CD25^+$ T cells or $TGF\beta$ in GM plant-mediated AAD. Adoptive transfer of $CD4^+CD25^+$ T cells from SSA-lupin-fed mice to naive mice did not confer protection from AAD following SSA sensitization and i.t. challenge (results not shown). Furthermore, we observed no detectable $TGF\beta$ in PBLN supernatants from lupin or SSA-lupin-fed SSA-challenged mice (results not shown). Previous investigations have also reported that $TGF\beta$ does not significantly contribute to oral tolerance-mediated suppression of Th2 cell-mediated AAD (13).

The development of plant-derived biopharmaceuticals for the treatment of disease processes is an emerging field in vaccine development (26, 66). Recent clinical and experimental investigations using models of viral and bacterial infection have shown a significant degree of success in using GM plants as a vaccine approach for the treatment and prevention of diseases (29, 31, 32, 67–69). Current conventional allergen immunotherapy techniques are expensive to prepare, require multiple injections of soluble allergen over a period of years, and have had limited success (6, 70). Indeed, early vaccination for immunomodulation to Ag-specific type 1 or suppressive responses has been proposed as a means

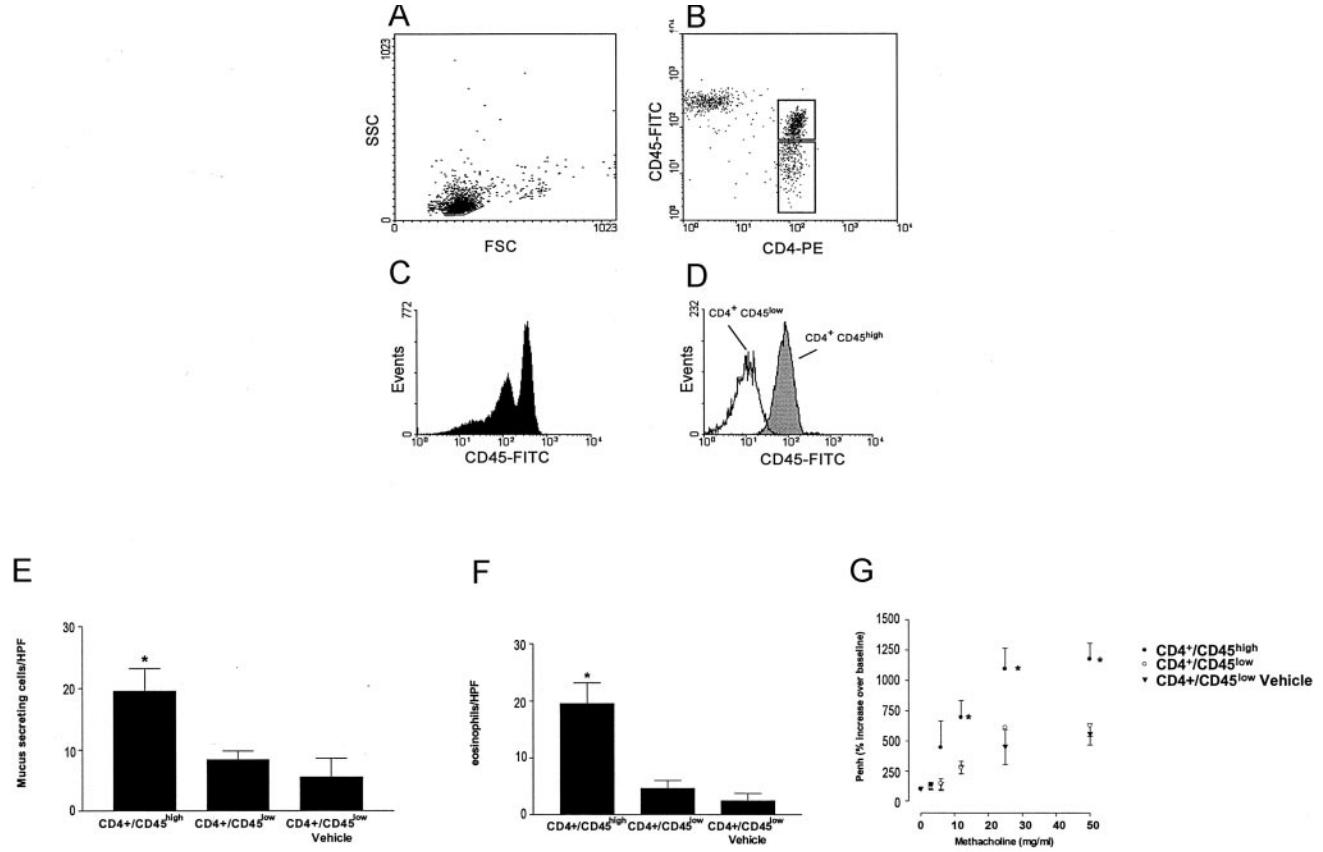

FIGURE 9. Characterization of the role of $CD4^+CD45^{high}$ and $CD4^+CD45^{low}$ T cells in GM plant-induced suppression of AAD. *A–D*, Representative side light scatter vs forward light scatter dot plot of mesenteric lymph node cells from SSA-lupin-fed mice. *B*, Representative histogram plot showing levels of CD45 expression on gated $CD4^+$ PE cells from mesenteric lymph node cells from SSA-lupin-fed mice. *C*, Representative CD4 PE vs CD45 FITC dot plot of mesenteric lymph node cells from SSA-lupin-fed mice. The boxes depicted are the gate stringencies for purification of the $CD4^+CD45^{low}$ and $CD4^+CD45^{high}$ populations. *D*, Representative histogram of the purified $CD4^+CD45^{low}$ and $CD4^+CD45^{high}$ T cell populations. The purity of both $CD4^+CD45^{high}$ T cells and of $CD4^+CD45^{low}$ T cells was >95% on reanalysis. *E*, Quantification of mucus-secreting cell numbers in lung tissue from SSA- or vehicle-challenged mice receiving purified $CD4^+CD45^{high}$ or $CD4^+CD45^{low}$ T cells. Mucus-secreting cell numbers in lung tissue were determined, as described in *Materials and Methods*. Seven similar fields were counted per lung. Data are the mean mucus-secreting cell numbers/HPF ± SEM from $n = 3$–5 mice per group from duplicate experiments. *F*, Quantification of eosinophil numbers in lung tissue from SSA- or vehicle-challenged mice receiving purified $CD4^+CD45^{high}$ or $CD4^+CD45^{low}$ T cells. Eosinophil numbers in lung tissue were determined in SSA- or vehicle-challenged mice, as described in *Materials and Methods*. Seven similar fields were counted per lung. Data are the mean eosinophil numbers/HPF ± SEM from $n = 3$–5 mice per group from duplicate experiments. *G*, AHR in response to β-methacholine was determined in SSA- or vehicle-challenged mice receiving purified $CD4^+CD45^{high}$ or $CD4^+CD45^{low}$ T cells. Data shown represent the mean Penh (% increase over baseline) ± SEM (where $n = 8$–10) from duplicate experiments. Cumulative dose-response curve to methacholine (1, 3, 6, 12, 24, and 50 mg/ml) challenge was measured by barometric plethysmography, and the data represent the percentage increase in Penh over baseline reactivity. Statistical significance ($p < 0.05$) of differences was determined using Student's unpaired *t* test.

of prophylaxis against allergic diseases (71). Thus, the use of GM plants containing appropriate allergens may be a useful approach for the establishment of a specific type 1 or suppressive response in young individuals.

One of the limitations in the development of GM plants for biopharmaceuticals has been the expression level of recombinant foreign proteins. In general, levels of recombinant proteins produced by transgenic plants range from 0.001 to 1% of total soluble protein (72). These low levels of expression of recombinant protein are thought to contribute to the low Ag-specific protective Ab titers (32, 66). In the present study, we have used a legume seed storage protein promoter (Pea vicillin) to target the expression and concentration of SSA in the legume seeds (73). We obtained significant levels of SSA in the legume seeds reaching 5% of total extractable seed protein (73). The high levels of SSA expression in the lupin may explain why we achieved a robust Ab and protective T cell response in the mice.

In summary, we have shown that plant-based vaccine immunotherapy with SSA-lupin dramatically suppressed the development of SSA-specific experimental asthma, including mucus hypersecretion, eosinophilic inflammation, and AHR. We show that oral ingestion of a GM plant containing a potential allergen can suppress the production of Th2-type Abs (IgG1) specific to that allergen. Furthermore, we show that the response is critically dependent on the development of $CD4^+CD45RB^{low}$ suppressor T cell population and the production of IFN-γ. Our findings are the first demonstration that GM plants expressing an allergen can suppress AAD and suggest that plant-based vaccine therapy may be a feasible potential therapeutic strategy for protection against allergic diseases.

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