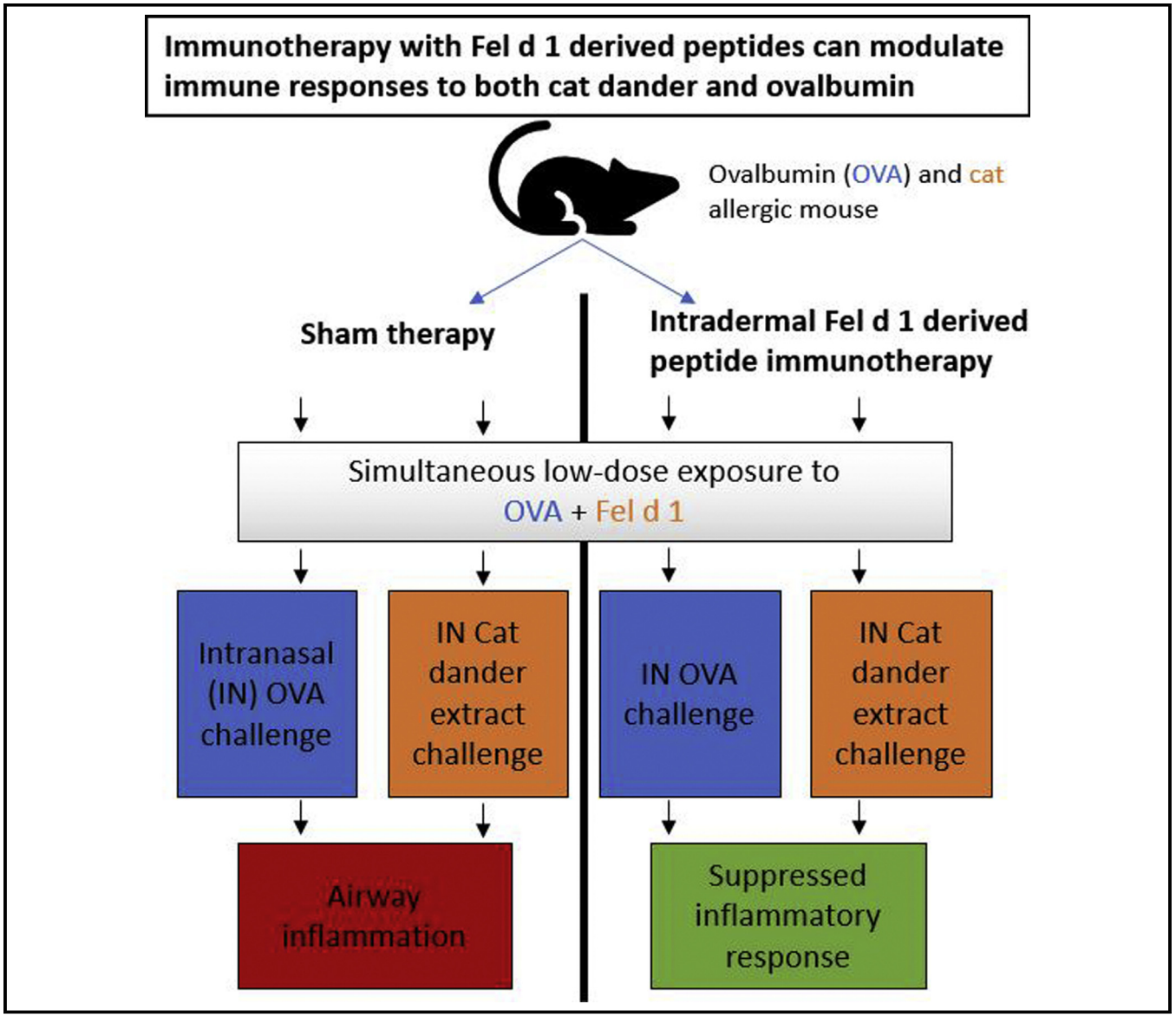
Induction of bystander tolerance and immune deviation after Fel d 1 peptide immunotherapy

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GRAPHICAL ABSTRACT



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Disclosure of potential conflict of interest: M. Larché reports grants and personal fees from Adiga Life Sciences and personal fees from and stock ownership in Circassia/Circassia Pharmaceuticals during the conduct of the study and personal fees from UCB, personal fees from Allergopharma GmbH & Co KG, and personal fees from Aravax and ownership interest in Adiga Life Sciences outside the submitted work; in addition, M. Larché is an inventor on 17 patent families (pending or issued) held by Circassia. The rest of the authors declare that they have no relevant conflicts of interest.

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Background: Treatment of patients with cat allergy with peptides derived from Fel d 1 (the major cat allergen) ameliorated symptoms of cat allergy in phase 2 clinical trials.

Objective: We sought to demonstrate that the tolerance induced by Fel d 1 peptide immunotherapy can be exploited to reduce allergic responses to a second allergen, ovalbumin (OVA), in mice sensitized dually to OVA and Fel d 1.

Methods: Induction of tolerance to OVA was achieved through simultaneous exposure to both allergens after peptide treatment. Functional tolerance to each allergen was assessed in a model of allergic airways disease in which treated mice were protected from eosinophilia, goblet cell hyperplasia, and T_H2 cell infiltration.

Results: Suppression of allergic responses to cat allergen challenge was associated with significant increases in numbers of $CD4^+CD25^+Foxp3^+$ T cells, IL-10^+ cells, and $CD19^+$IL-10^+ B cells, whereas the response to OVA was associated with a marked reduction in numbers of T_H2 cytokine–secreting T cells and less prominent changes in outcomes associated with immune regulation.

Conclusions: These observations suggest that immune tolerance induced by peptide immunotherapy can be used experimentally to treat an allergic response to another allergen and that the molecular mechanisms underlying induction of tolerance to a treatment-specific allergen and a bystander allergen might be different. (J Allergy Clin Immunol 2018;■■■:■■■-■■■.)

Key words: *Allergy, asthma, peptide, immunotherapy, ovalbumin, cat dander, T_H2, regulatory T cell, intermolecular tolerance*

Abbreviations used
APC: Allophycocyanin
BAL: Bronchoalveolar lavage
Breg: Regulatory B
CDE: Cat dander extract
dLN: Draining lymph node
FoxP3: Forkhead box P3
HDM: House dust mite
OVA: Ovalbumin
PE: Phycoerythrin
SIT: Specific immunotherapy

Sensitivity to cats is one of the most common forms of allergy and has been associated with severe asthma, wheezing in children, and morbidity.[1-3] Allergic disease is typified by the presence of allergen-specific IgE in the sera of patients,[4] the presence of increased T_H2 cytokine levels and T_H2 cell counts,[5,6] and compromised immune regulation.[7-10] By modifying the underlying immunologic cause of allergic disease, specific immunotherapy (SIT) is capable of preventing the development of asthma in children with seasonal allergic rhinoconjunctivitis[11] and reducing sensitizations to other allergens[12,13] and is known to exhibit therapeutic benefit extending beyond the treatment period.[14] Despite the clear benefits of SIT, an estimated 5% to 35% of patients receiving SIT experience systemic adverse events[15] because of the ability of allergens to cross-link allergen-specific IgE on the surfaces of mast cells and basophils.

A recent double blind, placebo-controlled phase II study has shown that immunotherapy performed with Cat-SPIRE (Synthetic Peptide Immuno Regulatory Epitopes; peptides derived from Fel d 1, the major cat allergen) significantly improves symptoms in patients with cat allergy[16] for 2 years after treatment.[17] Importantly, the incidence of treatment-related adverse events was no different than with placebo,[18] which likely reflects that the peptides used lack the size and requisite 3-dimensional structure to cross-link IgE bound to FcεRI receptors.[19,20]

The mechanisms by which treatment with allergen-derived CD4 T-cell epitopes can induce antigen-specific tolerance to the entire allergen are poorly understood. It has been postulated that peptide treatment modifies allergen-specific T-cell responses to generate cells with inhibitory or regulatory function that can then be exploited to confer tolerance on other cells in the same local environment, a so-called "tolerogenic environment."[21] Indeed, peptide immunotherapy has been reported to induce linked-epitope suppression in this way. Treatment of patients with cat allergy with Fel d 1–derived peptide epitopes induced suppression of PBMC responses specific for not only the treatment peptides but also other Fel d 1 epitopes that were not included in the treatment preparation.[22] However, in the same experiments no changes were observed in response to an unrelated antigen (purified protein derivative of *Mycobacterium tuberculosis*), indicating that the suppression induced by peptides is not a systemic phenomenon. This conclusion is supported by safety data in several hundred subjects treated with peptide immunotherapy in human clinical trials. A period of simultaneous presentation of both treatment and nontreatment epitopes by the same antigen-presenting cell is likely required to facilitate linked epitope suppression, which might occur by default when the 2 epitopes are within the same allergen.[23]

We demonstrated previously that suppression/regulation can also occur between 2 different allergens if they are coadministered.[24] Specifically, mice sensitized dually to ovalbumin (OVA) and house dust mite (HDM) were intranasally treated with synthetic peptides derived from Der p 1, the major HDM allergen. Der p 1 peptide immunotherapy reduced airway hyperresponsiveness, eosinophilia, and goblet cell hyperplasia during a subsequent OVA allergen challenge.[24]

Patients with cat allergy typically have allergic sensitization to multiple allergens. Thus it would be therapeutically advantageous if the suppression generated by Fel d 1 peptides in patients with cat allergy could be exploited to suppress responses to other allergens. Although our finding that Der p 1 peptide therapy could suppress the OVA response in dually sensitized mice lends support to this idea, HDM is a very different allergen from Fel d 1, as evidenced by its ability to sensitize mice in the absence of adjuvant.

Another key difference between the clinical administration of Fel d 1 peptides and HDM peptides in the previous study is the route used to administer the peptides. Patients with cat allergy receive intradermal peptide therapy, whereas the mice with HDM and OVA allergy inhaled Der p 1 peptides. The route of peptide administration is important because mice tend to mount tolerogenic responses to inhaled peptides, whereas human subjects do not.[25,26]

Thus, given the differences between clinical studies with Fel d 1 and the HDM/OVA model of therapy, it remains unclear whether the tolerance to cat dander induced by Fel d 1 peptide

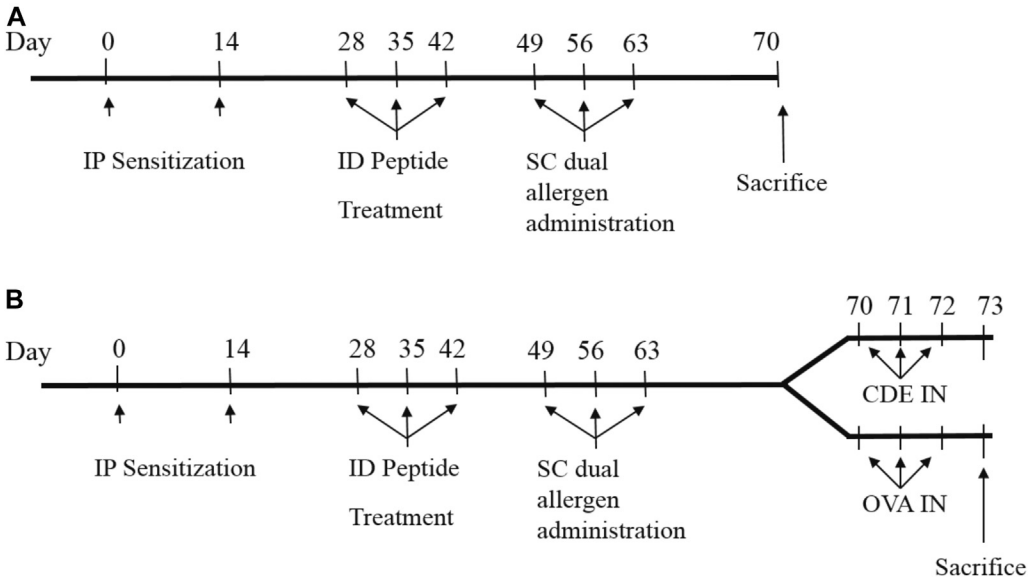


FIG 1. Allergen exposure and peptide therapy protocol. Mice were sensitized to OVA (100 μg) and Fel d 1 (1 μg) through 2 intraperitoneal injections (days 0 and 14). Three intradermal treatments of Fel d 1–derived peptides were administered (days 28, 35, and 42), which were followed by 3 subcutaneous exposure to both allergens (Fel d 1 and OVA; days 49, 56, and 63). Sham-treated mice received peptide vehicle (PBS) administered intradermally. Mice were killed 1 week later to assess the humoral and proliferative immune response **(A)** or after a series of intranasal allergen challenges (**B**; days 70, 71, and 72) with either OVA or CDE to assess the functional response. Mice were killed 24 hours after final challenge for measurement of airway responsiveness and cell procurement. *ID*, Intradermal; *IN*, intranasal; *IP*, intraperitoneal; *SC*, subcutaneous.

immunotherapy administered intradermally can be used therapeutically to ameliorate sensitization to other allergens. We have addressed this outstanding question in the present study by treating with intradermal Fel d 1 peptides, followed by subcutaneous coadministration of OVA and Fel d 1 proteins, to mice sensitized dually to cat and OVA and assessing their effect on both the cat and OVA response. Importantly, the intradermal Fel d 1 peptides together with the subcutaneous OVA and Fel d 1 proteins constituted the treatment regimen in this study.

METHODS

Animals

Female BALB/c mice aged 6 to 8 weeks were purchased from Charles River Laboratories (Saint-Constant, Quebec, Canada) and housed in specific pathogen-free conditions. Environmental control, a 12-hour light/dark schedule, and *ad libitum* access to food and water were maintained throughout the study. Animals were acclimatized to the facility for 1 week before the beginning of studies. All procedures were approved by the Animal Research Ethics Board at McMaster University and performed in accordance with the "Guide for humane use and care of laboratory animals."

Allergen materials and peptides

Lyophilized cat dander extract (CDE; Greer Laboratories, Lenoir, NC) and Grade V OVA (Sigma, St Louis, Mo), as well as purified Fel d 1 protein (Indoor Biotechnologies, Charlottesville, Va), were purchased and diluted in Dulbecco PBS to the required concentrations. Allergens were stored and frozen at −20°C. Treatment peptides were Fel d $1_{23\text{-}38}$ (EQVAQYKALPVVLENA) and Fel d $1_{29\text{-}45}$ (KALPVVLENARILKNCVDAK). These 2 peptides have been shown to elicit recall responses in BALB/c mice (unpublished data). These peptides do not have homology with the amino acid sequence of OVA. Peptides were synthetically produced (GL Biochem, Shanghai, China) by using standard Fmoc chemistry and purified by using high-pressure liquid chromatography (>95% purity). Lyophilized peptides were reconstituted in acidified water (10^{-4} mol/L HCl) and frozen (−80°C) before use.

Allergen sensitization, challenge, and peptide therapy

BALB/c mice (n = 4-5 per group performed twice) received an intraperitoneal prime and boost dual-allergen sensitization to OVA and Fel d 1 on days 0 and 14 (1 μg of Fel d 1 and 10 μg of OVA in alum per intraperitoneal administration). Mice were treated 3 times, once per week, with peptides derived from Fel d 1 (days 28, 35, and 42). Control groups (positive and negative) received PBS (peptide vehicle), whereas treatment groups received either 1 μg or 0.1 μg of both peptides administered intradermally. After a 1-week rest, mice were exposed simultaneously to Fel d 1 (0.5 μg) and OVA (0.01 μg) at subimmunogenic doses (data not shown) over a series of 3 subcutaneous injections (days 49, 56, and 63). After the sensitization and treatment protocols, examination of lymphocyte proliferative responses and humoral responses was performed (Fig 1, *A*) or the functional response to allergen was investigated through an intranasal allergen challenge (Fig 1, *B*). CDE (25 μg of protein) or OVA (100 μg) airway challenge was performed through 3 intranasal instillations of 25 μL. Negative control mice received PBS rather than allergen throughout. In addition to 1 μg of Fel d 1 and 10 μg of OVA, triple allergen–sensitized mice received 10 μg of timothy grass allergen in alum on days 0 and 14. Twenty-four hours after final challenge, tissues and samples were collected to assess the inflammatory response.

Sample collection and cell isolation

Bronchoalveolar lavage (BAL) was performed by injecting 2 aliquots (250 μL) of PBS into the lungs of tracheotomized mice. Aliquots were steeped for 5 seconds and then removed and pooled. BAL fluid was centrifuged subsequently (150*g*), and cells were enumerated and then prepared for cytology by means of cytocentrifugation (Cytospin 3 Centrifuge; Shandon Scientific, Sewickley, Pa). BAL fluid cells were differentiated based on

morphologic criteria and classified as either eosinophils, neutrophils, macrophage/monocytes, or lymphocytes by a blinded investigator after Wright-Giemsa staining. Blood was collected form a cardiac puncture, allowed to clot, and centrifuged (400*g*) to collect serum. BAL fluid supernatants and sera were stored at −20°C.

Draining lymph nodes (dLNs; mediastinal and peribronchial lymph nodes) and lungs were collected after exsanguination. Cells were released from the tissues by means of physical disruption (dLNs) or a 1.5-hour enzyme digestion at 37°C (lung) by using 300 U/mL of Type 1 collagenase (purified from *Clostridium histolyticum*; Worthington Biochemical, Lakewood, NJ) and 50 U/mL of DNase I (Sigma). Resultant cell suspensions were washed, and red blood cells were lysed (by using ACK lysis buffer), washed twice, and suspended in RPMI media (10% FBS, 1% penicillin/streptomycin, and 0.1% 2-mercaptoethanol) for enumeration.

Lung histology

Lung histology and morphometry were performed and quantified, as described previously.[27,28] Briefly, 3-μm transverse sections of the paraffin-embedded left lung lobe were taken after dehydration and formalin fixation. Hematoxylin and eosin and periodic acid–Schiff staining was performed to determine tissue eosinophilia and goblet cell hyperplasia, respectively. Images of first-generation airways were collected at ×40 or ×20 magnification and analyzed for eosinophils per square millimeter and goblet cells per millimeter, respectively, with a digital image analysis system (Northern Eclipse; Empix Imaging, Mississauga, Ontario, Canada).

ELISA

An ELISA MAX Mouse IgE kit (BioLegend, San Diego, Calif) was used to assay serum IgE levels. A BD OptEIA Mouse IgG2a ELISA set (BD Biosciences, San Diego, Calif) was used to assay serum IgG_{2a} concentrations. Kits were used according to the manufacturer's instructions. Allergen-specific assays were created by coating assay wells with 10 μg of allergen protein in coating buffer rather than capture antibody.

Flow cytometry

Cells were stained with antibody for 30 minutes after washing in staining buffer and blockade of Fc receptors. After staining, cells were fixed in 1% paraformaldehyde before analysis. Cells to be stained for intracellular cytokines were stimulated for 6 hours in phorbol 12-myristate 13-acetate (20 ng/mL; Sigma-Aldrich), ionomycin (1 μM; Sigma-Aldrich), and brefeldin A (10 μg/mL; Sigma-Aldrich); surface stained; permeabilized; and then stained for intracellular cytokines for 30 minutes. Forkhead box P3 (FoxP3) intracellular staining was performed by using the Regulatory T Cell Staining Kit (eBioscience, San Diego, Calif), according to the manufacturer's instructions. Antibodies used included fluorescein isothiocyanate–T1ST2 (CDJ8; MD Biosciences, Oakdale, Minn), phycoerythrin (PE)– and peridinin-chlorophyll-protein complex (PerCP)–CD4 (RM4-5; BD Biosciences), PerCP-CD8 (53-6.7; BD Biosciences), allophycocyanin (APC)–CCr4 (2G12; BioLegend), PE-Cy7–CD69 (H1.2F3; eBioscience), APC-Cy7–CD3 (17A2; BD Biosciences), APC- and PE-CD25 (PC61.5; eBioscience), PE-Cy7–CD19 (1D3; eBioscience), PE–Siglec-F (E50-2440; BD Biosciences), APC-Gr1 (RB6-BC5; eBioscience), APC–IL-10 (JES5-16E3; BD Biosciences), APC–IFN-γ (XMG1.2; BD Biosciences), Alexa Fluor 488–IL-13 (eBio13A; eBioscience), PE–IL-4 (11B11; BD Biosciences), APC–IL-5 (TRFK5; BD Biosciences), and PE-Cy7–IL-17A (eBio17B7; eBioscience). Flow cytometric analysis was performed with the FACSCanto II (Becton Dickinson) and FlowJo software (TreeStar, Ashland, Ore).

Tritiated thymidine incorporation assay

Proliferation to pretitrated allergen (CDE, OVA, or timothy grass) concentrations was assessed in triplicate. Splenocyte (2.5×10^5) and dLN (2×10^4) cells were cultured (at 37°C in a 5% CO_2 atmosphere at 95% relative humidity) in a flat-bottom, 96-well microtiter plate (BD Falcon) for 5 days. On day 6, the supernatant was harvested and replaced with fresh culture media (RPMI supplemented with 10% FBS and 1% penicillin/streptomycin; Invitrogen, Carlsbad, Calif). On culture day 7, cells were pulsed with 0.05 μCi of tritiated ([*methyl*-^{3}H]) thymidine (General Electric Healthcare, Bale d'Urfe, Quebec, Canada). Sixteen hours later, plates were frozen (−20°C) and then harvested (FilterMate Universal Harvester; PerkinElmer, Woodbridge, Ontario, Canada) onto glass-fiber filter mats (Wallac, Turku, Finland).

Scintillation fluid (BetaPlate Scint; PerkinElmer) was added to the filter mats, and resultant light emissions were counted for 1 minute by using a MicroBeta Trilux (PerkinElmer).

Data analysis

Data are presented as means ± SEMs. All comparisons were 2-tailed, and *P* values of less than .05 were considered significant. ANOVA and *post hoc* Bonferroni-corrected *t* tests were used to determine significance.

RESULTS

Coadministration of Fel d 1 and OVA is required for Fel d 1 peptide immunotherapy to suppress the OVA response

Having previously demonstrated that peptide therapy can facilitate induction of tolerance beyond the treated allergen,[24] we sought to develop a systemic model of dual-allergen sensitization to Fel d 1 and OVA to assess whether treatment with Fel d 1–derived peptides could suppress responses to OVA. Briefly, mice were sensitized to OVA and Fel d 1 in alum by using a prime-boost intraperitoneal injection series and were treated with 3 subcutaneous administrations of 2 synthetic Fel d 1–derived peptides (at either 0.1 or 1 μg) to induce tolerance to Fel d 1. After peptide therapy, mice were exposed to OVA and Fel d 1 subcutaneously to induce tolerance to OVA. Sham-sensitized and treated animals had low levels of IgE (Fig 2, *A*) and IgG_{2a} (Fig 2, *B* and *C*). Dual allergen–sensitized, sham-treated mice exhibited significant T_H2 immune polarization observed as increased total serum IgE levels (Fig 2, *A*). Treatment of dual-sensitized animals with 1 μg of Fel d 1–derived peptides significantly reduced serum IgE levels (Fig 2, *A*) while also increasing cat-specific IgG_{2a} levels (Fig 2, *B*). Importantly, administration of both allergens (CDE and OVA) elicited bystander tolerance as OVA-specific IgG_{2a} levels were increased significantly (Fig 2, *C*).

We next sought to assess whether bystander tolerance was due to a specific immune response or nonspecific immune suppression arising from Fel d 1 peptide therapy. In animals that received subcutaneous OVA but not Fel d 1, after Fel d 1 peptide therapy, IgE levels and OVA-specific IgG_{2a} levels remained the same as those in untreated mice (see Fig E1, *A* and *B*, in this article's Online Repository at www.jacionline.org). Additionally, dual allergen–sensitized animals that received peptide therapy but a sham subcutaneous injection (diluent alone) possessed an unaltered proliferative response to OVA *in vitro* (see Fig E1, *C*). The specificity of tolerance generated by therapy with synthetic Fel d 1–derived peptides and simultaneous allergen exposure was assessed further by using triple-sensitized animals. Splenocytes derived from animals sensitized to timothy grass, Fel d 1, and OVA retained a significant proliferative response to *in vitro* stimulation with timothy grass after treatment with Fel d 1–derived peptides and simultaneous exposure to OVA/CDE (see Fig E1, *D*). Therefore induction of bystander tolerance appeared to have been restricted to the allergens administered

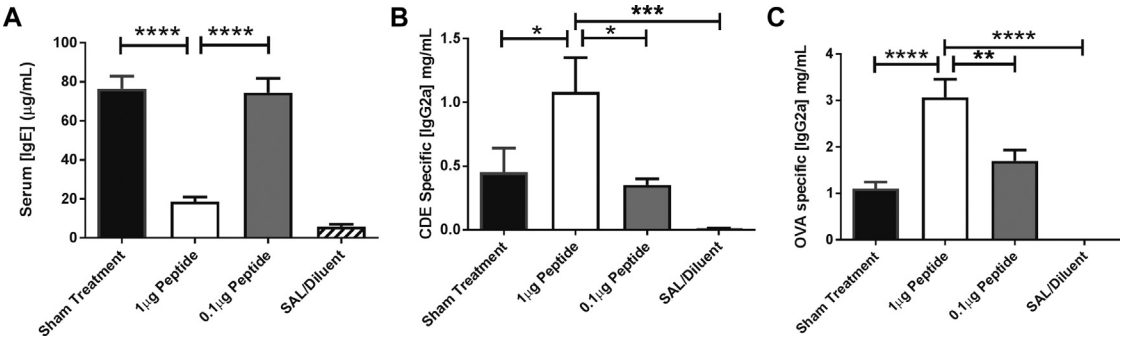


FIG 2. Fel d 1–derived peptide immunotherapy reduces antibody and lymphocyte proliferative responses. Concentrations of serum IgE **(A)**, cat dander–specific IgG_{2a} **(B)**, and OVA-specific IgG_{2a} **(C)** were assessed by means of ELISA, as described in the Methods section. Mice were sensitized to both Fel d 1 and OVA and treated with Fel d 1–derived peptides before exposure to both allergens subcutaneously. Groups are as follows: sensitized and sham treated *(Sham Treatment)*, sensitized and treated with 1 μg of each peptide *(1μg Peptide)*, sensitized and treated with 0.1 μg of each peptides *(0.1μg Peptide)*, and sham sensitized and treated *(Sal/Diluent)*. All data are presented as means ± SEMs of 2 independent experiments pooled together (n = 10 per group). $*P \leq .05$, $**P \leq .01$, $***P \leq .001$, and $****P \leq .0001$ compared with 1 μg of peptide by using an ANOVA and the *post hoc* Bonferroni-corrected Student *t* test.

simultaneously during the subcutaneous administration after peptide immunotherapy. However, further experiments would be required to definitively establish the antigen specificity of the bystander effect.

Fel d 1 peptide immunotherapy followed by dual subcutaneous allergen administration ameliorates CDE-induced lung inflammation in dual allergen–sensitized mice

After our finding that Fel d 1 therapy followed by dual subcutaneous allergen administration exposure reduces both cat- and OVA-specific humoral immunity, we investigated whether this was associated with functional tolerance to both allergens by performing an *in vivo* allergen challenge.

Mice were sensitized to both allergens and peptide treated, followed by dual administration of allergen proteins, and then challenged intranasally with CDE (Fig 1, *B*). Resultant cellular infiltrates in BALF fluid, peribronchial tissue (histology), and whole lung tissue (flow cytometry) were assessed. Intranasal challenge with CDE in dual allergen–sensitized and sham-treated animals induced marked cellular infiltration into BAL fluid (Fig 3, *A*) that was composed primarily of eosinophils and neutrophils (Fig 3, *B*). Low-dose peptide therapy significantly reduced both total BAL fluid cell infiltrates and eosinophilia by up to 50%. After CDE intranasal challenge, lung tissue eosinophilia was assessed by using flow cytometry ($Siglec\text{-}F^{+}CD11c^{+}$)[29] and histology (hematoxylin and eosin staining). Sensitized mice receiving a sham treatment possessed significantly increased levels of eosinophils in lung tissue (Fig 3, *C*) and the peribronchial space (Fig 3, *D-H*). In contrast, low-dose Fel d 1 peptide–treated mice were protected from peribronchial eosinophil accumulation (Fig 3, *D*).

Previously, it was demonstrated that peptide immunotherapy can reduce goblet cell hyperplasia, a common feature of allergic airways disease.[22] CDE challenge of sham-treated and allergen-sensitized mice demonstrated a greater than 100-fold increase in goblet cell density compared with saline-treated mice (Fig 3, *I-M*). High-dose peptide therapy reduced goblet cell hyperplasia by 30% (Fig 3, *I-M*).

OVA-induced allergic airways disease can be regulated by Fel d 1 peptide immunotherapy and dual-allergen administration

Having determined that Fel d 1 peptide immunotherapy could protect mice from challenge with CDE, we proceeded to investigate whether tolerance could be conferred to OVA-induced responses. To address this question, dual allergen–sensitized and Fel d 1 peptide–treated mice were given 3 intranasal OVA challenges after simultaneous subcutaneous exposure to OVA and CDE.

In dual allergen–sensitized, sham-treated mice, 3 intranasal OVA challenges induced robust cellular infiltration into the lumen of the lungs (Fig 4, *A*), with the infiltrate consisting primarily of eosinophils (Fig 4, *B*). Peptide immunotherapy with 1 μg of Fel d 1–derived peptides significantly reduced total BAL fluid cellular infiltration, and a trend toward a reduction in numbers of BAL fluid eosinophils was observed ($P = .07$; Fig 4, *A* and *B*).

Analysis of whole lung eosinophilia after OVA challenge confirmed that sham-treated, allergen-sensitized mice had robust lung tissue eosinophilia. Peptide therapy induced a dose-dependent reduction in lung tissue eosinophilia of up to 50% (Fig 4, *C*). Further examination of the eosinophilic response demonstrated that peribronchial eosinophilia induced by OVA challenge was significantly reduced with 1 μg of peptides and dual-allergen administration (Fig 4, *D-H*). Thus the dose of peptides that significantly improved responses to CDE (Fig 3, *D*) differed from the dose required to suppress OVA responses (Fig 4, *D*). OVA challenge in sensitized, sham-treated mice induced robust goblet cell hyperplasia that was inhibited significantly in peptide/dual subcutaneous allergen–treated mice (Fig 4, *I-M*).

Fel d 1 peptide immunotherapy and dual-allergen administration reduces OVA-induced inflammatory cytokine production

Successful clinical studies of peptide immunotherapy in allergic subjects have been associated routinely with reduced inflammatory cytokine production by $CD4^{+}$ T cells.[30-36] We

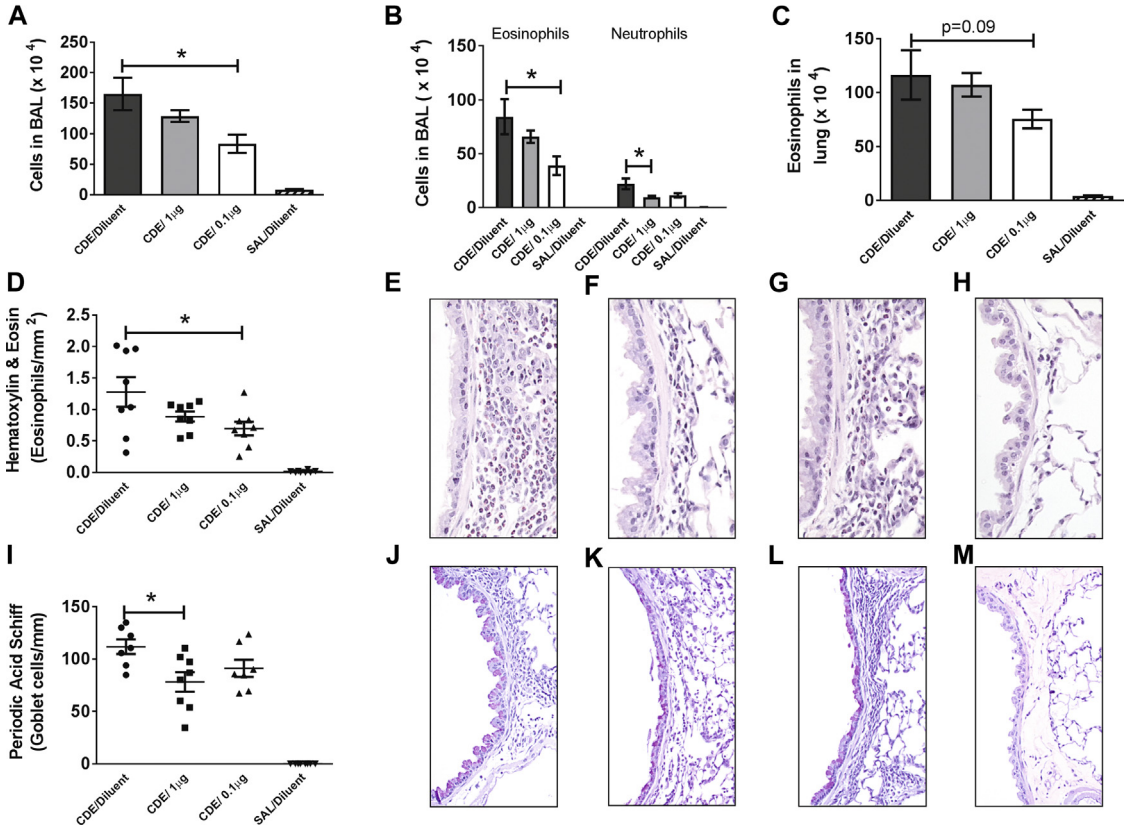


FIG 3. Fel d 1 peptide immunotherapy ameliorates CDE challenge–induced cellular recruitment to the lung. Mice (n = 8 per group from 2 pooled experiments) were sensitized to Fel d 1 and OVA and then treated with 1 μg *(/1μg)* or 0.1 μg *(/0.1 μg)* of Fel d 1–derived peptides or PBS *(/Diluent)*. Following the therapeutic protocol, mice were challenged intranasally with 25 μg of CDE *(CDE/)* or saline *(SAL/)*. **A,** Total BAL fluid cells were enumerated and differentiated by using Wright-Giemsa staining. **B,** BAL fluid eosinophils and neutrophils. **C,** Lung cells were isolated, and eosinophilia was determined by using flow cytometry (sialic acid–binding immunoglobulin-type lectin [Siglec]$^{-}$F^{+}Gr1^{-}) after recall challenge. **D,** Peribronchial eosinophilia was determined by using hematoxylin and eosin staining of formalin-fixed lungs. Representative histologic images of stained sections at ×20 magnification are shown: **E,** CDE/Diluent; **F,** CDE/1 μg; **G,** CDE/0.1 μg; and **H,** SAL/Diluent. **I,** Goblet cell hyperplasia was determined by periodic acid–Schiff staining of formalin-fixed lungs. Representative images of stained lung sections at ×10 magnification are shown: **J,** CDE/Diluent; **K,** CDE/1 μg; **L,** CDE/0.1 μg; and **M,** SAL/Diluent. Data are presented as means ± SEMs with or without individual points. *$P \leq .05$, as assessed by using ANOVA and the *post hoc* Bonferroni-corrected Student *t* test.

sought to assess whether Fel d 1 immunotherapy could similarly reduce T_H2 polarization of CD4^{+} T cells in OVA-challenged mice, as assessed by using flow cytometry.

Examination of T_H2 polarization of lung T cells (assessed as T1/ST2^{+} T cells[37-39]) after therapy revealed that OVA challenge of dual-sensitized, sham-treated mice induced a significant T_H2 polarization of lung T cells (Fig 5, *A*). Immunotherapy with Fel d 1 peptides and dual-allergen administration significantly reduced the number of T1/ST2^{+} T cells by approximately 50% (Fig 5, *A* and *B*). After assessment of T_H2-polarized cells in the lungs of treated mice, the ability of T cells to produce archetypal T_H2 cytokines was assessed. CD4^{+} T cells derived from the lungs of sham-treated mice contained a significantly larger number of cells staining positively for IL-4, IL-5, and IL-13, as determined by using flow cytometry (Fig 5, *C-E*). Peptide/dual-allergen treatment significantly reduced the number of IL-4^{+} and IL-5^{+} T cells and resulted in a nonsignificant approximately 33% reduction in IL-13^{+} T cells. Together, these data indicate that Fel d 1 immunotherapy and dual-allergen exposure modulated the T_H2 immune response to OVA.

To corroborate these data, we examined the prototypical T_H2 antibody IgE to assess whether reductions in T_H2 polarity affected the B-cell compartment. Sham-treated mice possessed increased levels of OVA-specific IgE that were significantly reduced by peptide therapy (Fig 5, *F*).

Interestingly, in sensitized and treated mice rechallenged with CDE rather than OVA, reductions in T_H2 polarization were less notable. Although CDE challenge of peptide-treated, dual-allergic mice reduced T_H2 T-cell polarization (see Fig E2, *A* and *B*, in this article's Online Repository at www.jacionline.org), modulation of cytokine production was less apparent, with no significant reduction in numbers of T cells producing IL-4^{+} (see Fig E2, *C*), IL-5^{+} (see Fig E2, *D*), or IL-13^{+} (see Fig E2, *E*) T cells.

Reduction of inflammatory response to OVA is associated with increased numbers of IL-10^{+} T and B cells

Previously, we have shown a dependency of peptide immunotherapy on the presence of IL-10 in a murine model of

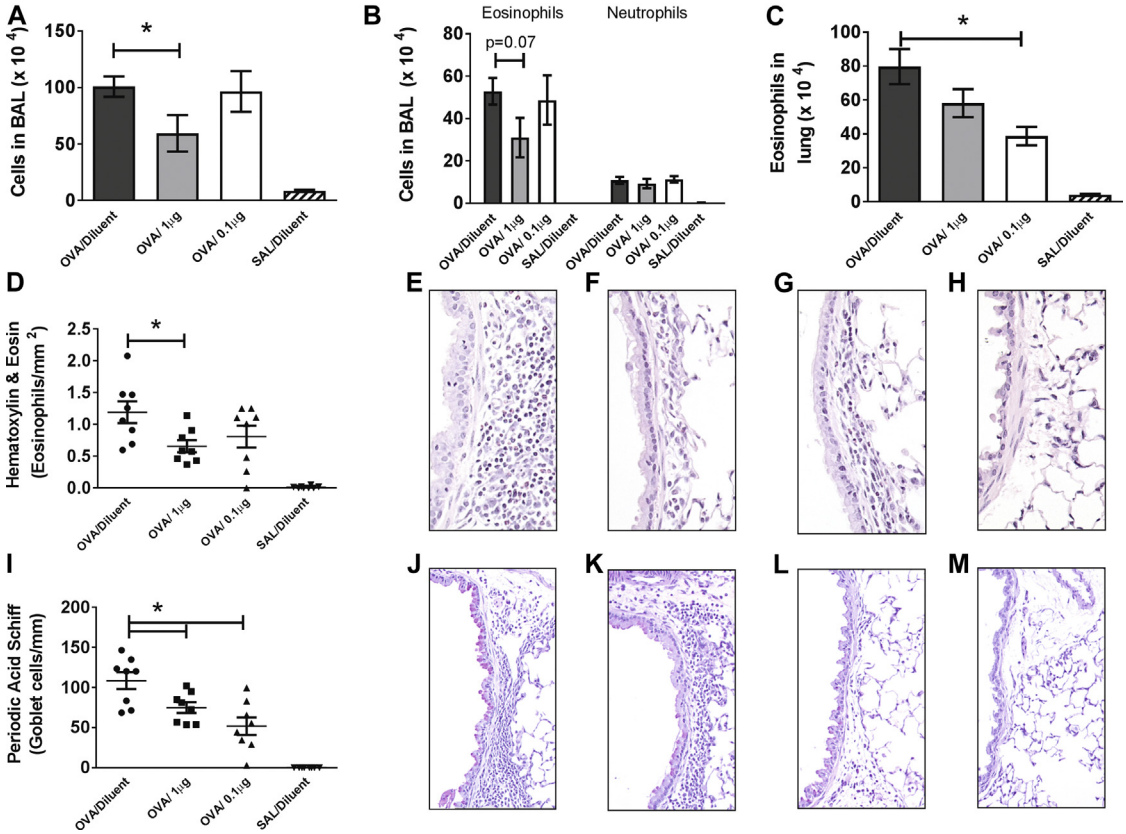


FIG 4. Fel d 1 peptide immunotherapy prevents OVA-induced lung inflammation. Mice (n = 8 per group from 2 pooled experiments) were sensitized to Fel d 1 and OVA and then treated with 1 μg *(/1μg)* or 0.1 μg *(/0.1μg)* of Fel d 1–derived peptides or PBS *(/Diluent)*. Following the therapeutic protocol, mice were challenged intranasally with 100 μg of OVA *(OVA/)* or saline *(SAL/)*. **A,** Total BAL fluid cells were enumerated and differentiated by using Wright-Giemsa staining. **B,** BAL fluid eosinophils and neutrophils. **C,** Lung cells were isolated, and eosinophilia was determined by using flow cytometry (sialic acid–binding immunoglobulin-type lectin [Siglec]$^{-}$F^{+}Gr1^{-}) after recall challenge. **D,** Peribronchial eosinophilia was determined by using hematoxylin and eosin staining of formalin-fixed lungs. Representative histologic images of stained sections at ×20 magnification are shown: **E,** OVA/Diluent; **F,** OVA/1 μg; **G,** OVA/0.1 μg; **H,** and SAL/Diluent. **I,** Goblet cell hyperplasia was determined by using periodic acid–Schiff staining of formalin-fixed lungs. Representative images of stained lung sections shown at ×10 magnification are shown: **J,** OVA/Diluent; **K,** OVA/1 μg; **L,** OVA/0.1 μg; and **M,** SAL/Diluent. Data are presented as means ± SEMs with or without individual points. *$P \leq .05$ assessed by using ANOVA and the *post hoc* Bonferroni-corrected Student *t* test.

cat allergy[22] and an association between IL-10 production and induction of bystander tolerance. We investigated whether IL-10 production was increased after treatment. Lung and dLN cells were analyzed for IL-10 expression after OVA allergen challenge. The total number of IL-10^{+} lung digest cells was reduced modestly (although not significantly) after therapy compared with sham-treated animals (Fig 6, *A*). In contrast to lung-derived cells, numbers of IL-10–producing CD4^{+}CD25^{+} cells in dLNs were increased significantly after peptide/dual-allergen immunotherapy ($P > .05$; Fig 6, *B*).

We further characterized the regulatory phenotype of treated mice by quantifying FoxP3, a transcription factor that controls regulatory T-cell development.[40] Analysis of FoxP3 expression in T cells from digested lungs of OVA-challenged mice revealed that both sensitized mice and treated mice had increased numbers of FoxP3^{+} cells compared with unsensitized control mice (Fig 6, *C* and *D*). Finally, the intensity of FoxP3 staining within CD4^{+} T cells, as quantified by mean fluorescence intensity, significantly increased with peptide therapy (Fig 6, *E*).

In addition to assessing IL-10 and FoxP3 expression after OVA challenge, we assessed the regulatory phenotype of mice receiving a CDE challenge. In the lungs and dLNs of CDE-challenged mice, peptide/dual-allergen immunotherapy dose-dependently and significantly increased IL-10 expression (see Fig E3, *A* and *B*, in this article's Online Repository at www.jacionline.org) compared with that seen in sham-treated mice. Moreover, quantification of FoxP3 expression after peptide immunotherapy revealed that CDE-challenged mice had significantly more FoxP3^{+} T cells (see Fig E3, *C* and *D*) and an increased abundance of FoxP3 in those cells (see Fig E3, *E*).

Regulatory B (Breg) cells have been identified in murine models as key mediators of peripheral tolerance[41] and have been demonstrated recently to be capable of preventing and/or resolving allergic airway inflammation.[42] We investigated whether Fel d 1 peptide immunotherapy was capable of inducing Breg cells. Treatment with 1 μg of Fel d 1–derived peptides followed by subcutaneous dual-allergen administration led to a significant increase in the CD19^{+}IL-10^{+} cell population within

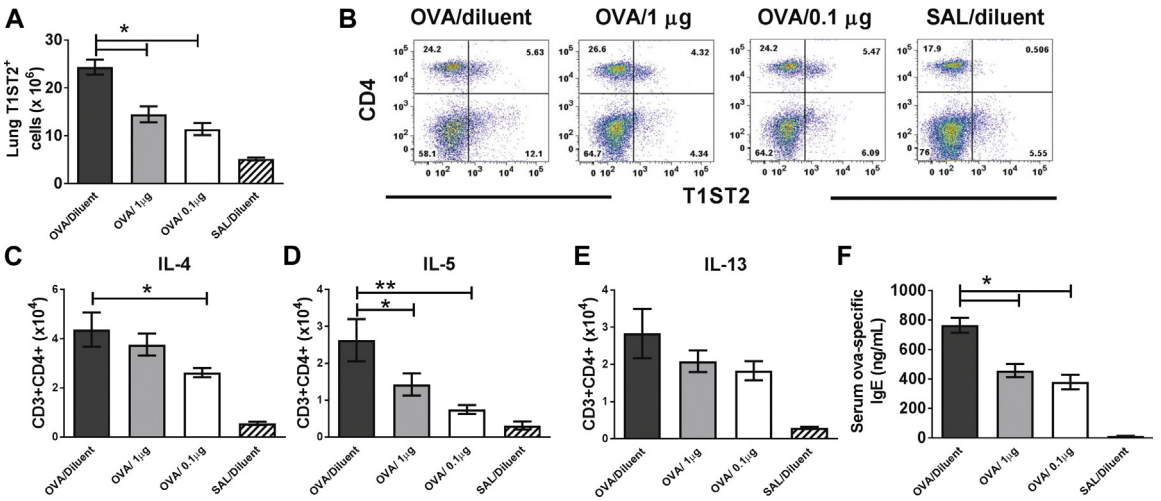


FIG 5. Reduced T_H2 cell recruitment and cytokine expression after OVA rechallenge in Fel d 1–derived peptide-treated mice. Mice (n = 8 per group from 2 pooled experiments) were sensitized to Fel d 1 and OVA and then treated with 1 μg *(/1 μg)* or 0.1 μg *(/0.1 μg)* of Fel d 1–derived peptides or PBS *(/Diluent)*. Following the therapeutic protocol, mice were challenged intranasally with 100 μg of OVA *(OVA/)* or saline *(SAL/)*. The T_H2 phenotype of cells derived from lungs of allergen-challenged mice was assessed. **A,** Expression of T1ST2 in lymphocytes. **B,** Average percentage of $CD4^+T1ST2^+$ cells is depicted within representative dot plot images of a typical flow cytometric experiment. Groups were ordered as follows: OVA/Diluent, OVA/1μg, OVA/0.1μg, and SAL/Diluent. **C-E,** Lung cells were stimulated for 6 hours with phorbol 12-myristate 13-acetate and ionomycin in the presence of brefeldin A, at which point the expression of IL-4 (Fig 5, *C*), IL-5 (Fig 5, *D*), and IL-13 (Fig 5, *E*) were examined within the T-cell ($CD3^+CD4^+$) population. **F,** Concentration of OVA-specific IgE was assessed by using ELISA, as described in the Methods section. Data are presented as means ± SEMs. $*P \leq .05$ and $**P \leq .01$, as assessed by means of ANOVA and the *post hoc* Bonferroni-corrected Student *t* test.

the lungs of mice compared with sham-treated animals (see Fig E3, *F*). However, the accumulation of Breg cells after OVA challenge was also assessed and found to be unchanged compared with that in sham-treated animals (data not shown).

Fel d 1 peptide/dual-allergen administration induces immune deviation

In addition to our investigation of the induction of regulatory T-cell and B-cell responses, we assessed whether peptide immunotherapy had induced immune deviation toward T_H1 by examining IFN-γ production. Sensitized sham-treated mice had a modest increase in numbers of $CD4^+IFN\text{-}\gamma^+$ cells after OVA challenge. In mice receiving low-dose peptide therapy, numbers of $CD4^+IFN\text{-}\gamma^+$ cells were significantly increased by nearly 2-fold, whereas therapy with 1 μg of peptide induced a nonsignificant ($P = .06$) 33% increase in numbers of $CD4^+IFN\text{-}\gamma^+$ cells (Fig 7, *A*). Furthermore, both tested doses of peptides doubled the percentage of T cells producing $IFN\text{-}\gamma^+$ cells ($P < .05$; Fig 7, *B*). Because these data support expansion of a T_H1 phenotype after peptide immunotherapy, we determined whether a similar effect could be observed in mice challenged with CDE. Indeed, treatment of cat allergen–sensitized mice with Fel d 1 peptides induced a T_H1 phenotype, as evidenced by a significantly increased abundance of $IFN\text{-}\gamma^+CD4^+$ T cells (see Fig E4, *A*, in this article's Online Repository at www.jacionline.org) and proportion of IFN-γ–producing CD4 cells (see Fig E4, *B*).

IL-17 is a proinflammatory non-T_H2 cytokine commonly expressed by T cells that has been linked to the inflammatory cascade observed in patients with allergic asthma,[43] severe asthmatic responses,[44] and T_H1 immunity.[45] We investigated IL-17 production after OVA challenge. Challenge of sham-treated mice with OVA induced a significant 8-fold increase in IL-17 production by activated lung-derived T cells; however, the absolute number of $IL\text{-}17^+$ cells was small (Fig 7, *C*). Treatment reduced numbers of $IL\text{-}17^+$ T cells by approximately 50%. In CDE-challenged mice numbers of $IL\text{-}17^+$ T cells appeared to be reduced, but this change did not reach statistical significance (see Fig E4, *C*).

DISCUSSION

In the present study we demonstrated that therapy with Fel d 1–derived peptides, followed by a period of dual-allergen exposure (CDE and OVA), can facilitate induction of bystander tolerance and inhibit OVA-induced inflammation. Treatment protected dual allergen–sensitized mice from eosinophilia, goblet cell hyperplasia, or T_H2 cellular infiltration on airway challenge with either CDE or OVA. Interestingly, immune responses to these 2 allergen sources were subtly different. Fel d 1 peptide–induced modulation of the response to cat allergen extract was associated with significant increases in numbers of $CD4^+CD25^+Foxp3^+$ T cells, $IL\text{-}10^+$ cells, and $CD19^+IL\text{-}10^+$ B cells. In contrast, the response to OVA, which was achieved through the Fel d 1 peptide therapy combined with subcutaneous coadministration of OVA and cat allergen extract, resulted in a marked reduction of T_H2 cytokine–secreting T cells (immune deviation) and less prominent changes to outcomes associated with immune regulation (eg, no association with $IL\text{-}10^+$ B cells). An important implication of these observations is that the mechanisms that give rise to bystander tolerance might be self-limiting

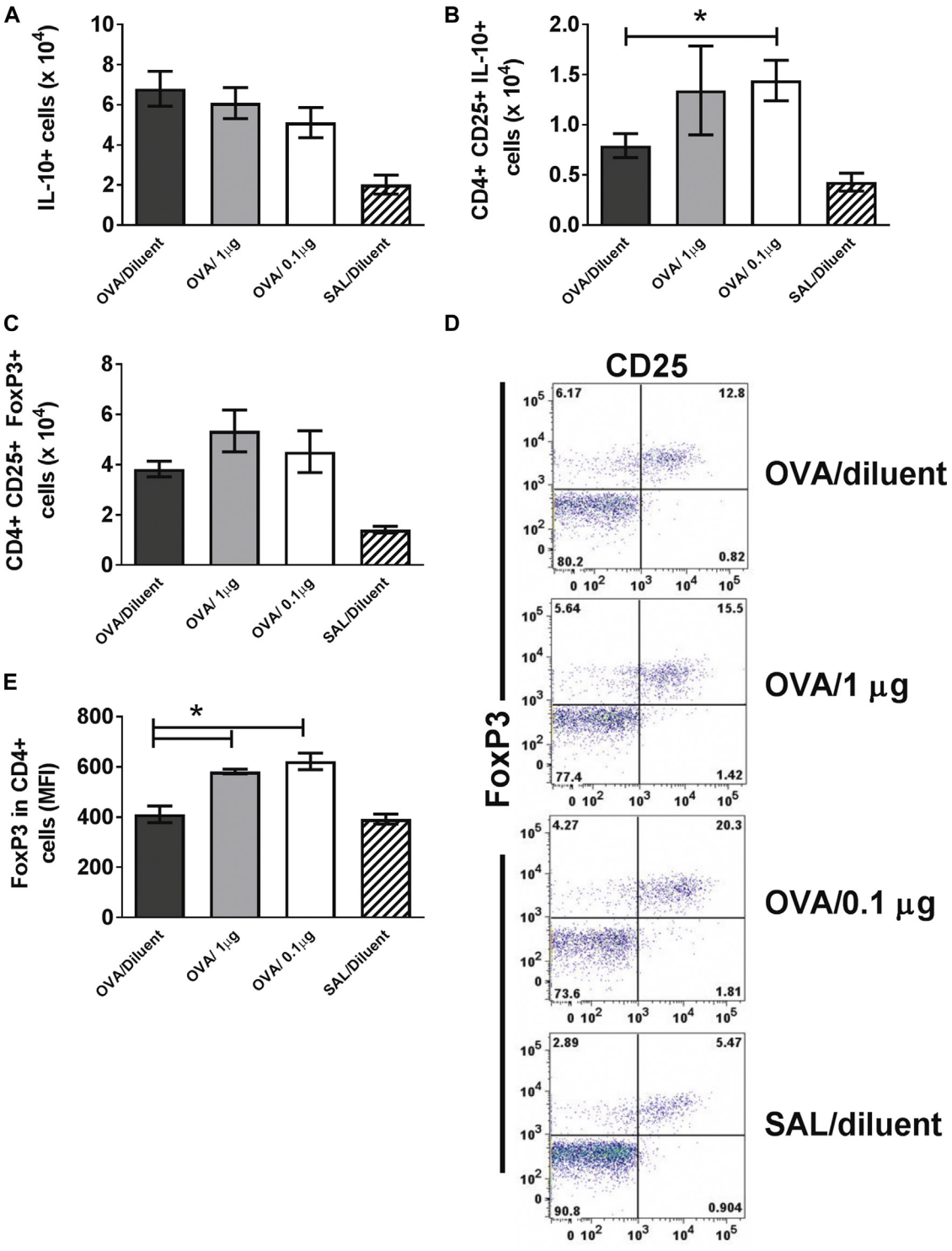


FIG 6. Mice challenged with OVA after Fel d 1 immunotherapy mount a moderate regulatory response. Twenty-four hours after intranasal OVA challenge, mice were killed, and isolated dLN and lung cells were stained for surface markers and intracellular cytokines expression. Mice (n = 8 per group from 2 pooled experiments) were sensitized to Fel d 1 and OVA and then treated with 1 μg *(/1 μg)* or 0.1 μg *(/0.1 μg)* of Fel d 1–derived peptides or PBS *(/Diluent).* Following the therapeutic protocol, mice were challenged intranasally with 100 μg of OVA *(OVA/)* or saline *(SAL/).* **A,** Total IL-10$^+$ lung cells. **B,** Total IL-10$^+$CD25$^+$CD4$^+$ dLN cells. **C,** Total FoxP3$^+$CD25$^+$CD4$^+$ cells within the lungs. Lung lymphocytes, gated on CD4, were further analyzed for their FoxP3 and CD25 expression. **D,** Average percentage of positive cells is depicted within representative dot plots, with the panels in descending order as follows: OVA/Diluent, OVA/1μg, OVA/0.1μg, and SAL/Diluent. **E,** Magnitude of FoxP3 expression within lung CD4$^+$ cells, as assessed by using mean fluorescence intensity. Data are presented as means ± SEMs. *$P \leq .05$, as assessed by using ANOVA and the *post hoc* Bonferroni-corrected Student *t* test.

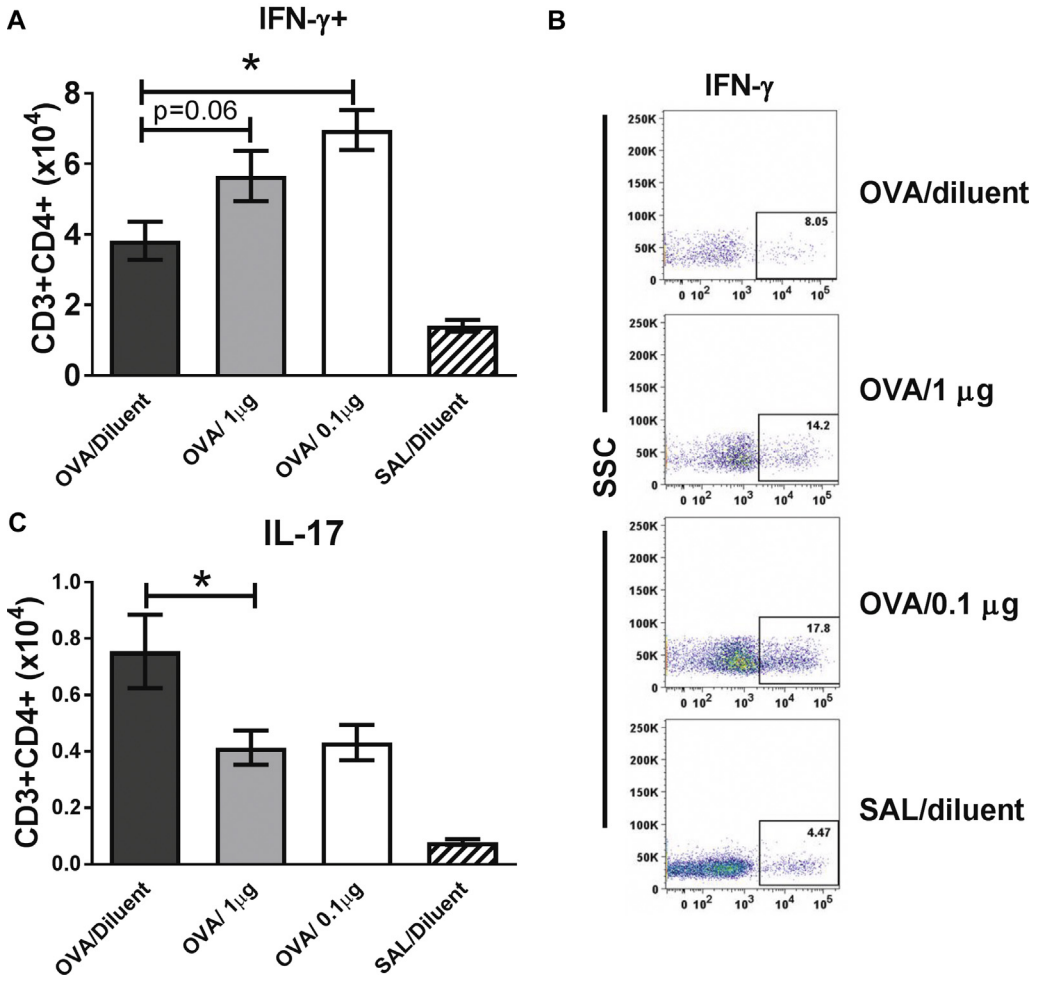


FIG 7. Fel d 1 immunotherapy induces immune deviation in OVA-challenged mice. Twenty-four hours after intranasal OVA challenge, mice were killed, and isolated dLN and lung cells were stained for surface markers and intracellular cytokine expression. Mice (n = 8 per group from 2 pooled experiments) were sensitized to Fel d 1 and OVA and then treated with 1 μg *(/1 μg)* or 0.1 μg *(/0.1 μg)* of Fel d 1–derived peptides or PBS *(/Diluent)*. Following the therapeutic protocol, mice were challenged intranasally with 100 μg of OVA *(OVA/)* or saline *(SAL/)*. **A,** Total $CD4^+$ T cells producing IFN-γ and percentage of lung lymphocytes, gated on CD4, producing IFN-γ are depicted in representative dot plots. **B,** Panels are presented in descending order as follows: OVA/Diluent, OVA/1 μg, OVA/0.1 μg, and SAL/Diluent. *SSC*, Side scatter. **C,** Enumeration of lung T cells producing IL-17. Data are presented as means ± SEMs. $*P \leq .05$, as assessed by using ANOVA and the *post hoc* Bonferroni-corrected Student *t* test.

(downregulation of T_H2 responses in this case), whereas peptide modulation of the specific response (characterized by induction of regulatory pathways; IL-10 and IL-10^+ B cells) might be capable of dominant suppression of nonspecific responses. Thus the immune response to antigen-specific therapy appears to have dominant regulatory capacity, whereas the immune response to the bystander protein is a terminal process. Taken together, these data indicate that different elements of immune regulation induced by peptide immunotherapy can be used experimentally to treat an allergic response to another allergen.

In ongoing clinical studies patients with cat allergy have been treated by using intradermal injection with an equimolar mixture of 7 Fel d 1–derived peptides. Phase 2 studies of this approach have demonstrated the ability of peptide immunotherapy to reduce responsiveness to provocation with complex mixtures of cat allergens contained in extracts.[16,17] Similar results have been achieved with grass pollen allergy.[46] The ability of peptides derived from one allergen protein to reduce responses to mixtures of multiple allergens raises the question of whether treatment with peptides from one molecule are able to create a localized tolerogenic environment in which tolerance or hyporesponsiveness can be induced to proteins from other simultaneously administered allergen sources. The results of this study demonstrate that peptides of Fel d 1 containing dominant T-cell epitopes can be used to create an environment that can be exploited to modulate the OVA response.

Despite extensive investigation of the pathogenesis of allergic diseases and improved understanding of the mechanisms of action of allergen immunotherapy, novel, safe, rapid, and effective therapeutics for allergic diseases have rarely reached the clinic.[46,47] Clinical trials have demonstrated that peptide immunotherapy for the treatment of patients with cat allergy–related rhinoconjunctivitis is well tolerated[18] and effective[16] and offers long-lasting protection.[17] Previously, we have

demonstrated that linked epitope suppression develops after peptide immunotherapy because subjects treated with a mixture of 12 Fel d 1 peptides displayed reduced proliferative and cytokine responses not only to the 12 treatment peptides but also to an additional 4 Fel d 1 peptides not included in the treatment phase.[22] The suppression in this model was not observed in responses to unrelated antigens that were not coadministered with the treatment antigen, which is in keeping with the findings of others.[48] Furthermore, in murine studies this phenomenon was determined to be dependent on IL-10, which is in agreement with other models of peptide-induced, T cell–mediated regulation.[49]

Previously, we described an example of bystander tolerance in which treatment with peptides from one protein elicited tolerance not only to the treatment protein but also to an unrelated protein. We observed a reduction in inflammatory responses to inhaled OVA challenge in OVA and HDM dual-sensitized mice that had been treated with intranasal HDM peptides.[24] To our knowledge, no clinical studies have directly and prospectively studied such phenomena. However, in a placebo-controlled study of 29 multiple allergen–sensitized children, pollen immunotherapy significantly reduced responsiveness to allergen challenge with HDM or cat allergen.[50] The extent to which these observations were dependent on simultaneous natural exposure to the nontreatment allergens was not addressed in this study, and thus the antigen specificity of the observed bystander phenomenon cannot be determined.

The current studies were designed to specifically investigate whether treatment with peptides from a single allergen protein create a local tolerogenic environment that could be exploited to suppress inflammatory responses to a second unrelated allergen. In addition, we wanted to determine whether this effect could be generated when treating animals intradermally, the same treatment route used in current clinical studies. The route of administration is relevant to clinical studies because asthmatic patients treated with intradermal peptides[34] but not inhaled peptides[26] were protected from subsequent challenge. Furthermore, because allergic patients are commonly polysensitized, we set out to use a model in which sensitization to both allergens was fully established before therapy rather than using a prophylactic treatment regimen that might misrepresent the clinical setting. This study focused on outcomes associated with allergic airways disease, such as airway eosinophilia and inflammatory T_H2 infiltration of the airways because these have been linked historically to allergic asthma.[51]

In our model of dual-allergen sensitization, challenge was associated with allergen-induced airway eosinophilia and/or goblet cell hyperplasia and other cardinal features of allergic airways disease. Treatment with Fel d 1–derived peptides and subcutaneous administration of both allergen proteins significantly suppressed both CDE- and OVA-induced lung inflammation, as shown through reductions in total BAL fluid cellularity, peribronchial eosinophilia, whole-lung eosinophilia, and goblet cell hyperplasia. Concomitant reductions in lung T_H2 cell-surface markers and numbers of cytokine expressing T cells were associated with resolution of OVA-specific (but not Fel d 1–specific) allergic responses, whereas increased expression of the prototypical T_H1 cytokine IFN-γ might implicate immune deviation as a means of regulation. Parameters related to regulatory responses, such as numbers of $CD4^+CD25^+Foxp3^+$ T cells, $IL\text{-}10^+$ cells, and $CD19^+IL\text{-}10^+$ B cells, were significantly increased after CDE but not OVA challenge.

Our findings are in accordance with and expand on 2 recent studies that examined both the antigen-specific and nonspecific effects of immunotherapy. Bouchaud et al[52] prophylactically treated mice with large peptides (>50 amino acids) derived from the HDM allergen Der p 2. Peptide-treated mice were protected from challenge with HDM, thereby demonstrating the ability of a single peptide to block inflammation induced by challenge with a complex allergen that contains multiple immunogenic epitopes. Furthermore, mice prophylactically treated with a Der p 2–derived peptide were moderately protected from Der f 2–induced airway hyperresponsiveness, as assessed by means of a methacholine challenge. This demonstrates that Der p 2–derived peptide therapy was able to affect the immune response to a distinct allergen, although whether this was through infectious tolerance or simply T-cell cross-reactivity is unknown.

Our data expand on this study by demonstrating that short peptides administered after sensitization and coupled with coadministration of whole allergen proteins (OVA and Fel d 1) can prevent the development of airway inflammation induced by a distinct, non–cross-reactive allergen. Chien et al[53] also investigated the non–antigen-specific effects of immunotherapy by demonstrating that feeding mice OVA after sensitization to β-lactoglobulin–conjugated OVA ameliorated airway inflammation induced by challenge with either OVA or β-lactoglobulin.

Our data corroborate the notion that immunotherapy can modulate both specific and non–antigen-specific effects while demonstrating that peptide immunotherapy, a clinically relevant therapy administered in a clinically relevant route, can perform this function. Furthermore, our study provides novel insight into the different immunologic mechanisms that underlie tolerance induced directly in an antigen-specific fashion versus indirectly through bystander tolerance.

Fel d 1 peptide therapy has been linked to expansion of regulatory T-cell activity within human and mouse $CD4^+$ T-cell populations.[36] However, expression of FoxP3 by such populations has been an unreliable surrogate marker, although in those studies the intensity of FoxP3 expression per cell was not evaluated. In a previous study we noted increased FoxP3 expression in T cells after peptide treatment.[24] In another model sustained suppression by $FoxP3^+$ T cells was required to observe the related phenomenon of infectious tolerance in which tolerant T cells direct other T cells to become regulators.[22,54] Thus induction of FoxP3-expressing populations or increased expression of FoxP3 per cell is often associated with induction of tolerance but is not a prerequisite.

In addition to expansion of T-cell populations with regulatory phenotypes after CDE challenge, we observed a 3-fold increase in numbers of IL-10–producing B cells, which have been described as Breg cells (B_R1/B10/Breg cells). This substantial population of B cells was observed after cat allergen challenge but not after OVA challenge. Thus these Breg cells might be a key differentiating mechanism of the dominant tolerance induced to the treatment allergen (Fel d 1 in this case) but not to the bystander allergen by peptide immunotherapy. Further detailed studies will be required to definitively define the contribution of these cells to peptide-induced tolerance. Breg cells have recently been acknowledged as potentially important modulators of peripheral tolerance but have not been described previously in the context of peptide immunotherapy. van de Veen et al[55] described the

induction of IL-10–secreting B cells after whole allergen immunotherapy for bee venom allergy. These cells exhibited potent allergen-specific suppressive function and were particularly associated with the production of IgG_4 antibodies, which are believed to have "blocking" activity by virtue of their ability to sequester allergen and prevent contact with IgE-laden mast cells and basophils.

Evidence suggests that the dose of allergen (and likely peptide as well) during therapy affects the mechanism of suppression because low-dose immunotherapy induces a profile of active suppression, whereas high-dose therapy relies on clonal deletion and anergy.[56-58] Relative to other murine models of immunotherapy, the current study used low doses of Fel d 1 peptides and was able to reduce the T_H2 pathology associated with OVA challenge, perhaps through active immune suppression associated with FoxP3, IL-10, and immune deviation. The effect of peptide dose during treatment requires further study.

In summary, using a model of dual-allergen sensitization and peptide immunotherapy, we have demonstrated that Fel d 1 peptides, when coupled with coadministration of both Fel d 1 protein and an unrelated protein (OVA), are capable of attenuating T_H2 inflammation within the murine allergic lung after inhaled allergen challenge with the unrelated protein allergen. Furthermore, we have confirmed that the local tolerogenic environment generated by peptide therapy can be exploited to confer tolerance to unrelated molecules. We show that tolerance specifically to the treatment allergen is associated with a mixed phenotype of enhanced regulatory profiles ($CD4^+CD25^+Foxp3^+$ T cells, IL-10^+ cells, and $CD19^+$IL-10^+ B cells), whereas tolerance to the unrelated allergen is associated more closely with reduction in T_H2 cytokine–secreting cells and lacks the Breg cell component. These results can enhance our understanding of how allergen-specific (peptide) immunotherapy appears to result in downregulation of immune responses to allergens beyond those used for treatment. The potential therapeutic advantages of such a phenomenon remain to be fully evaluated.

We thank Helen Neighbour for her contributions to study design.

Key messages

- **Allergen immunotherapy with peptides from one allergen can be manipulated to reduce allergic responses to a second (bystander) sensitizing allergen.**
- **Tolerance to the bystander allergen requires both peptide treatment and coadministration of the treatment allergen and the bystander allergen.**
- **The immunologic mechanisms underlying tolerance induced to the 2 allergens are subtly different.**

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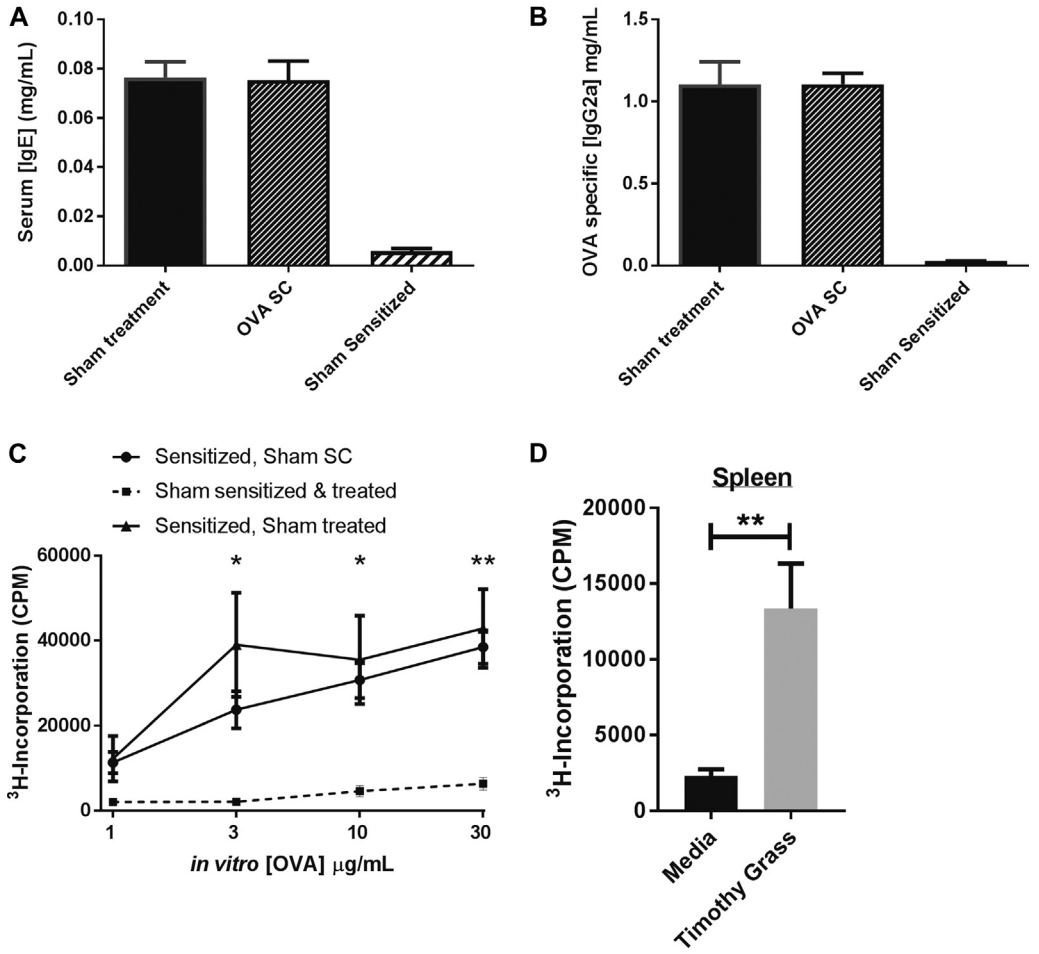


FIG E1. Fel d 1–derived peptide immunotherapy reduces antibody and lymphocyte proliferative responses. **A** and **B,** Concentrations of serum IgE (Fig E1, *A*) and OVA-specific IgG_{2a} (Fig E1, *B*) were assessed by means of ELISA, as described in the Methods section. **C,** Proliferation of splenocytes to OVA *in vitro* from sham-sensitized, sham-treated, and sham subcutaneous allergen exposed mice. **D,** Proliferation of splenocytes from triple allergen–sensitized animals (timothy grass, Fel d 1, and OVA) that received therapy with 0.1 μg of Fel d 1–derived peptides and simultaneous OVA and cat dander administered subcutaneously. Proliferation was assessed by using a tritiated thymidine incorporation assay. Data are presented as means ± SEMs of 2 independent experiments pooled together (n = 10 per group). *SC*, Subcutaneous. $^{*}P \leq .05$ and $^{**}P \leq .01$, Student *t* test.

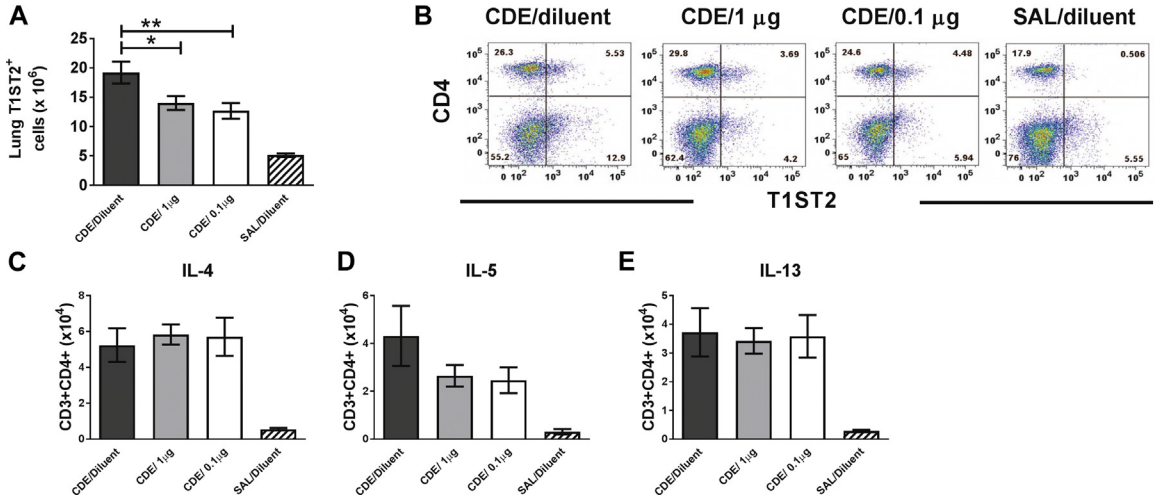


FIG E2. Fel d 1 peptide therapy prevents recruitment of T_H2 effector cells but not expression of T_H2 cytokines in the lungs of cat-challenged mice. Mice (n = 8 per group from 2 pooled experiments) were sensitized to Fel d 1 and OVA and then treated with 1 μg *(/1 μg)* or 0.1 μg *(/0.1 μg)* of Fel d 1–derived peptides or PBS *(/Diluent)*. Following the therapeutic protocol, mice were challenged intranasally with 25 μg of CDE *(CDE/)* or saline *(SAL/)*. The T_H2 phenotype of cells derived from the lungs of allergen-challenged mice was assessed. **A,** Expression of T1ST2 in lymphocytes. **B,** Average percentage of $CD4^+T1ST2^+$ cells is depicted within representative dot plot images of a typical flow cytometric experiment. Groups were ordered as follows: CDE/Diluent, CDE/1 μg, CDE/0.1 μg, and SAL/Diluent. **C-E,** Lung cells were stimulated for 6 hours with phorbol 12-myristate 13-acetate and ionomycin in the presence of brefeldin A, at which point expression of IL-4 (Fig E2, *C*), IL-5 (Fig E2, *D*), and IL-13 (Fig E2, *E*) was examined within the T-cell ($CD3^+CD4^+$) population. Data are presented as means ± SEMs. $*P \leq .05$ and $**P \leq .01$, as assessed by using ANOVA and the *post hoc* Bonferroni-corrected Student *t* test.

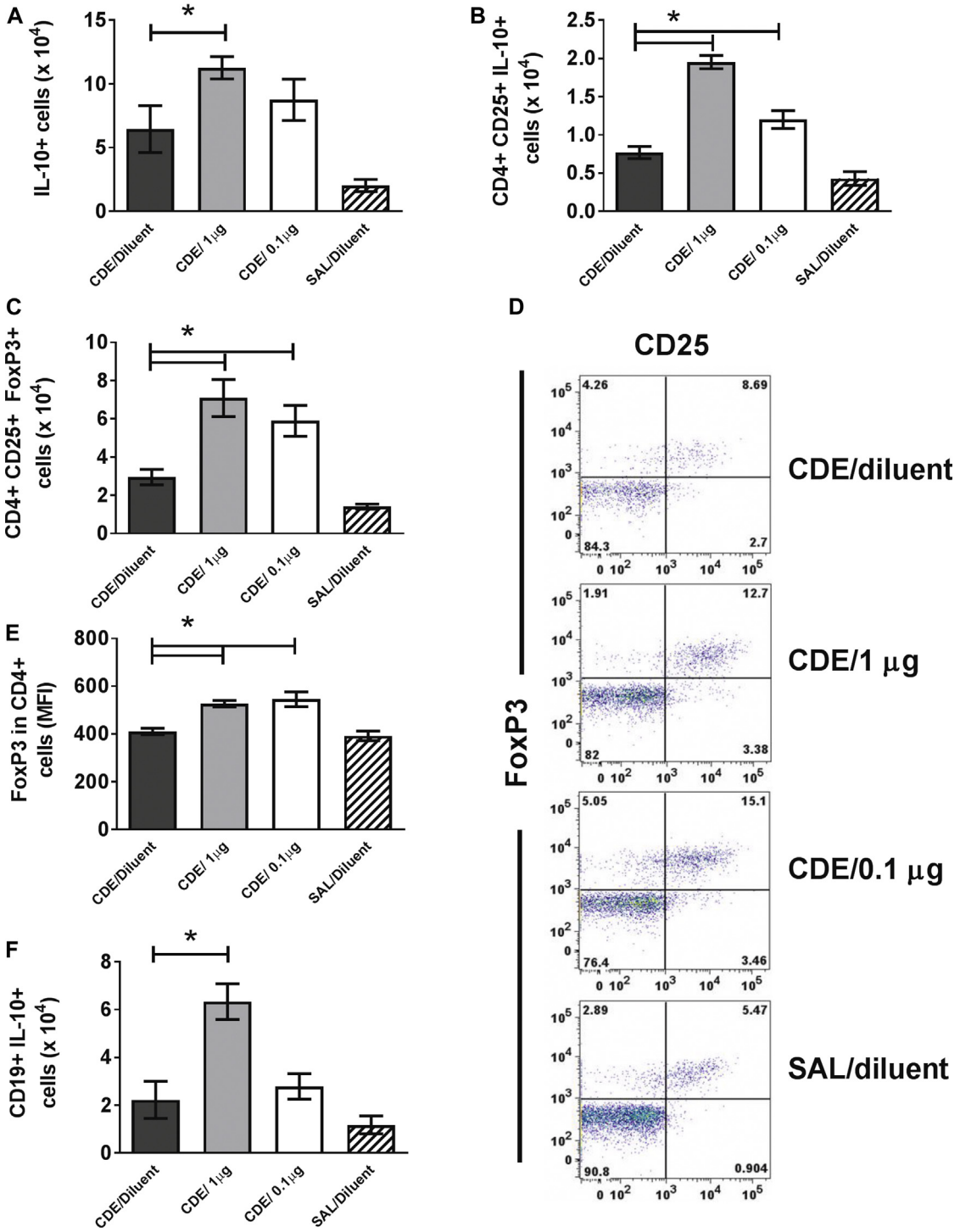


FIG E3. Fel d 1 immunotherapy allows manifestation of a regulatory response after CDE challenge. Twenty-four hours after intranasal OVA challenge, mice were killed, and isolated dLN and lung cells were stained for surface markers and intracellular cytokine expression. Mice (n = 8 per group from 2 pooled experiments) were sensitized to Fel d 1 and OVA and then treated with 1 μg *(/1 μg)* or 0.1 μg *(/0.1 μg)* of Fel d 1–derived peptides or PBS *(/Diluent)*. Following the therapeutic protocol, mice were challenged intranasally with 25 μg of CDE *(CDE/)* or saline *(SAL/)*. **A,** Total IL-10$^+$ lung cells. **B,** Total IL-10$^+$CD25$^+$CD4$^+$ dLN cells. **C,** Total FoxP3$^+$CD25$^+$CD4$^+$ cells within the lungs. **D,** Lung lymphocytes, gated on CD4, were further analyzed for their FoxP3 and CD25 expression. Average percentage of positive cells is depicted within representative dot plots, with the panels in descending order: CDE/Diluent, CDE/1 μg, CDE/0.1 μg, and SAL/Diluent. **E** and **F,** Mean fluorescence intensity of FoxP3 expression within lung CD4$^+$ cells (Fig E3, *E*) and numbers of CD19$^+$IL-10$^+$ cells (Fig E3, *F*) in the lung. Data are presented as means ± SEMs. *$P \leq .05$, as assessed by using ANOVA and the *post hoc* Bonferroni-corrected Student *t* test.

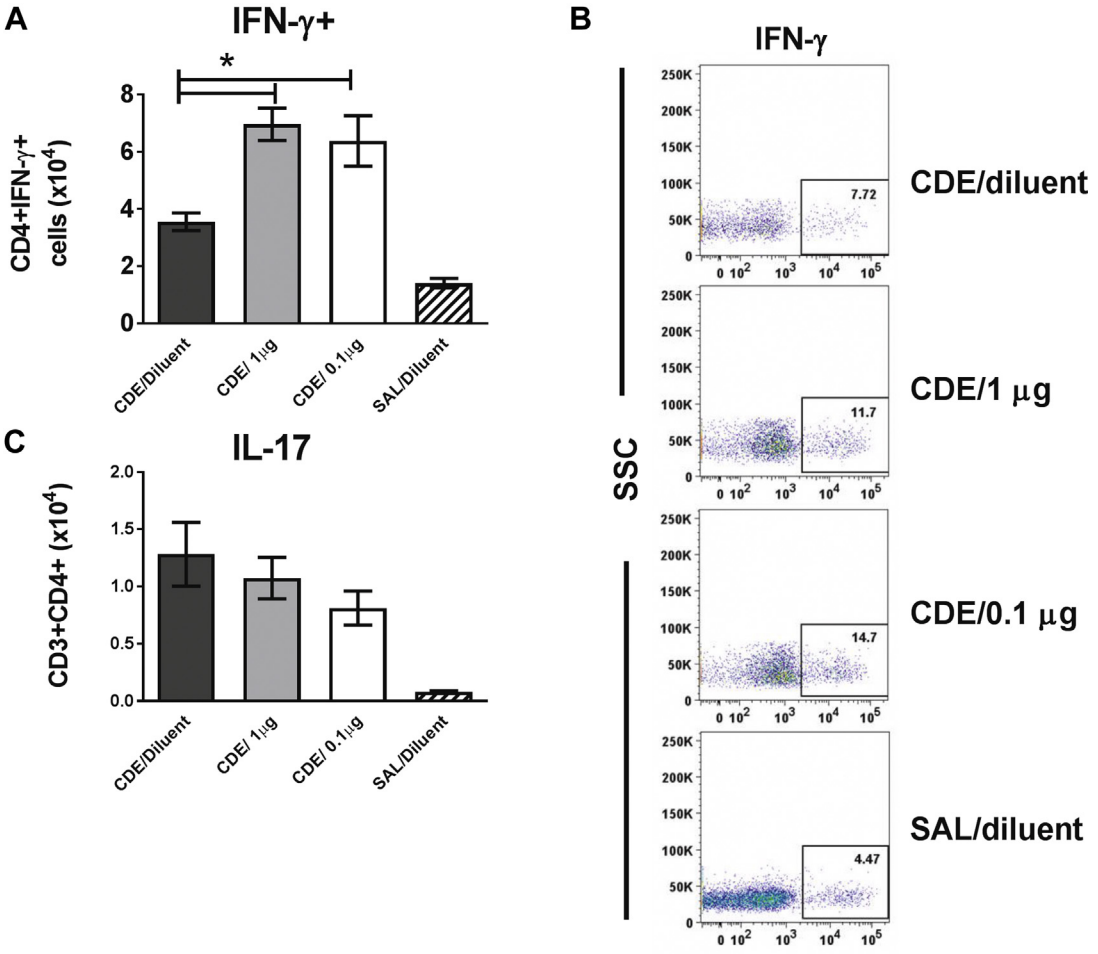


FIG E4. Fel d 1 immunotherapy allows manifestation of a regulatory response after CDE challenge. Twenty-four hours after intranasal OVA challenge, mice were killed, and isolated dLN and lung cells were stained for surface markers and intracellular cytokine expression. Mice (n = 8 per group from 2 pooled experiments) were sensitized to Fel d 1 and OVA and then treated with 1 μg *(/1 μg)* or 0.1 μg *(/0.1 μg)* of Fel d 1–derived peptides or PBS *(/Diluent)*. Following the therapeutic protocol, mice were challenged intranasally with 25 μg of CDE *(CDE/)* or saline *(SAL/)*. **A** and **B,** Total $CD4^+$ T cells producing IFN-γ and the percentage of lung lymphocytes, gated on CD4, producing IFN-γ are depicted in representative dot plots, with the panels in descending order: CDE/Diluent, CDE/1 μg, CDE/0.1 μg, and SAL/Diluent. **C,** IL-17 expression within T cells was probed after PMA and ionomycin stimulation. Data are presented as means ± SEMs. *$P \leq .05$, as assessed by using ANOVA and the *post hoc* Bonferroni-corrected Student *t* test.