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*J Immunol* 2008; 180:5360-5372; doi: 10.4049/jimmunol.180.8.5360

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Induction of Adaptive T Regulatory Cells That Suppress the Allergic Response by Coimmunization of DNA and Protein Vaccines

Huali Jin,* Youmin Kang,* Lin Zhao,* Chong Xiao,* Yanxin Hu,‡ Ruiping She,‡ Yang Yu,* Xiaogang Du,* Gan Zhao,* Terry Ng,§ Hsien-Jue Chu,§ and Bin Wang2*†‡

Allergen-induced immediate hypersensitivity (AIH) is a health issue of significant concern. This robust inflammatory reaction is initiated by the allergen-specific T cell responsiveness. Severe lesion reactions on skin are consequential problem requiring medical treatment. Effective Ag-specific treatments or prevention are lacking. Using a rodent model of AIH induced by flea allergens, we first report that coimmunization of DNA and protein vaccines encoding the flea salivary specific Ag-1 ameliorated experimental AIH, including Ag-induced wheal formation, elevated T cell proliferation, and infiltration of lymphocytes and mast cells to the site of allergen challenge. The amelioration of AIH was directly related to the induction of a specific population of flea antigenic specific T cells exhibiting a CD4+CD25+FoxP3+ phenotype, a characteristic of regulatory T (TREG) cells. These TREG cells expressing IL-10, IFN-γ, and the transcriptional factor T-bet after Ag stimulation were driven by a tolerogenic MHC class II+CD40low dendritic cell population that was induced by the coimmunization of DNA and protein vaccines. The tolerogenic dendritic cell could educate the naive T cells into CD4+CD25+FoxP3+ TREG cells both in vitro and in vivo. The study identified phenomenon to induce an Ag-specific tolerance via a defined Ag vaccinations and lead to the control of AIH. Exploitation of these cellular regulators and understanding their induction provides a basis for the possible development of novel therapies against allergic and related disorders in humans and animals. The Journal of Immunology, 2008, 180: 5360–5372.

T he disease allergen-induced immediate hypersensitivity (AIH) is considered harmful and persistent, both in humans and animals. Total flea extracts induce immediate intradermal allergic reactions in mice; therefore, this model system can be used to evaluate immunotherapeutic methods aimed at amelioration of AIH. Allergy is considered a consequence of persistent T cell activation, driving pathogenic inflammation against the host dermis by specific allergens. Several approaches are typically used to ameliorate AIH; and these include nonspecific immunosuppressive drugs or mAbs targeted to T or B cells (1, 2). However, this situation becomes tenuous, as such long-term treatment of recipients leads to their becoming immunocompromised and results in the loss of their ability to fight infections. Redirecting immunity from type Th2 to Th1 has also been attempted, but with limited success (3). A recent discovery of regulatory T (TREG) cells, including the naturally occurring thymus-derived CD4+CD25+ TREG cells (4–7), mucosal induced Th3 cells, and Ag-induced CD4+CD25− type 1 TREG cells (TREG1), has proposed their use as immunoregulators or suppressors of autoreactive pathogenesis (8). Various approaches have been explored to induce TREG cells to constrain the autoreactive T cells. In particular, induction of Ag-specific TREG cells targeted to allergy, asthma, and autoimmune disease Ags is considered a promising strategy. We recently demonstrated that induction of TREG cells is able to inhibit Ag-specific T cell function in vivo by co-inoculating Ag-matched DNA and protein Ags as coadministered vaccines (9). In this report, we further demonstrate that coimmunization of DNA and protein vaccines against flea allergic Ag induced suboptimal dendritic cells (DCs) that converted naive T cells into Ag-specific TREG cells to suppress the allergic response in vitro and in vivo. Exploitation of this cellular regulation response and its application to specific hyperimmune functions may present us with a potential therapeutic or even a prophylactic means to control unwanted immune disorders such as allergic dermatitis and other allergic diseases.

Materials and Methods

Mice and immunization

Adult female BALB/c and C57BL/6 mice (8–10 wk of age) from the Animal Institute of Chinese Medical Academy (Beijing, China) received pathogen-free water and food. The mice were immunized with total flea proteins (100 μg/mouse), plasmid pD100 (aa 66–80) encoding an epitope of FSA at 100 μg/mouse, or a mixture of proteins (100 μg) and a different dose of plasmid per mouse into the tibialis anterior muscle. All mice were immunized on day 0 and boosted on day 14.

Histology analysis

On day 14 following the second immunization, skin samples from mice in every group were collected within 30 min following the intradermal flea
Ag challenge, fixed in 4% of paraformaldehyde, and embedded in paraffin blocks. Sample skin sections were cut and fixed. Ag retrieval was accomplished by boiling the slides in 0.01 M citrate buffer (pH 6.0), followed by staining with H&E or toluidine blue for mast cells and analysis under a light microscope for determining histology changes (10). 

**Intradermal test (IDT)**

On day 14 after the last immunization, the mice were challenged intradermally with 1 μg/μl FSA (Greer Laboratories) on nonlesional lateral thorax skin. PBS is used as a sham control and histamine is used as a positive control. The diameter of the skin reaction was measured within 30 min after challenge by using a calibrated micrometer.

**DC culture**

DCs were cultured from mouse bone marrow. Briefly, bone marrow cell suspensions were cultured with RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% FBS and 20 ng/ml murine recombinant GM-CSF. At day 8, the CD11c+ DCs were purified by magnetic microbeads (R&D Systems).

**T cell recalled responses**

The CD4+ T cells isolated from immunized mice on day 14 were cultured at 5 × 10^6 cells/well in triplicate in 96-well plates containing RPMI 1640 (Invitrogen Life Technologies) with 10% FBS, and the APCs were bone marrow-derived DCs (1 × 10^5 cells/well) from the congenic mice. Cells were stimulated with 20 μg/ml FSA for 48 h. Following stimulation, cell proliferation was assessed by a colorimetric reaction after the addition of 20 μl of a novel tetrazolium compound and electron coupling reagent phenazine methosulfate (MTS-PMS; Promega) solution for 2–4 h; its color density was determined at 490 nm by plate reader (Magellan).

**Measurement of flea Ag-specific Abs**

Serum concentration of anti-flea IgG1 and IgE isotypes were measured using flea Ag-coated plates by ELISA and detection with specific HRP-conjugated rat anti-IgG1 and IgE Abs (Southern Biotechnology Associates); absorbance (450 nm) was measured using an ELISA plate reader (Magellan).

**RT-PCR**

Total RNA was isolated from spleen 14 days after the second immunization using TRIzol reagent (Promega). cDNA was synthesized, and PCR was performed with each of the following primers: HPR2, IL-2, IFN-γ, IL-4, IL-5, IL-13, IL-10, and IL-12 (11). For GATA3 and T-bet analysis, CD4+CD25+ T cells were isolated and RT-PCR was done using specific primer sequences as previously described (12).

**Isolation of CD4+CD25– T cells and adoptive transfer**

Single splenocyte suspensions were prepared from mouse spleen, and CD4+CD25– T cells were isolated and purified by using the MagCellect Mouse CD4+CD25– T Cell Isolation kit according to the manufacturer’s protocol (R&D Systems). The purity of the selected cell populations was 96–98%. The purified cells (1 × 10^6 per mouse) were adoptively transferred i.v. into C57BL/6 mice.

**MLR analysis**

CD4+ T cell populations were purified from C57BL/6 mice as described and were used as the responder cells. Stimulator cells were bone marrow-derived DCs from BALB/c mice and further treated with mitomycin C (50 μg/ml, Sigma-Aldrich) before use. The responder cells (1 × 10^6) were labeled with CFSE (5.0 μM; Molecular Probes) and cocultured with the DC stimulator cells (2 × 10^5) in 2 ml of medium. After 48 h, proliferation of the responder CD4+ T cells was analyzed using FACSCalibur and data were analyzed by gating of CFSE-positive cells with CellQuest Pro software (BD Biosciences).

**Coculturation of DCs and CD4+CD25– T cells in vitro**

DCs were isolated from the spleen of mice by magnetic microbeads (R&D Systems); the purification of DCs was performed with each of the following primers: HPR2, IL-2, IFN-γ, IL-4, IL-5, IL-13, IL-10, and IL-12 (11). For GATA3 and T-bet analysis, CD4+CD25– T cells were isolated and RT-PCR was done using specific primer sequences as previously described (12).
Statistical analysis

Two-group comparisons were statistically analyzed using the two-sided Student's t test. Three or more group comparisons were analyzed by the ANOVA followed by the Bonferroni test for comparing more than two groups. Differences were considered statistically significant with \( p < 0.05 \) and \( p < 0.01 \).

Results

Induction of AIH model in mice

AIH in animals can be induced after presensitization with flea extracts (14). The degree of AIH can be assessed by scoring the diameter of wheal or bleb at the challenge site using this IDT. As expected, preadministration of mice with flea extracts induced significant skin reactions (Fig. 1a), mast cell activation (Fig. 1b), coincident IgE production (Fig. 1c), and strong CD4+ T cell proliferation responses (Fig. 1d) in C57BL/6 mice, when compared with naive control animals following intradermal challenge. These data indicate the utility of this flea Ag-allergic model for the evaluation of novel therapeutic strategies directed against AIH.

Coimmunization of DNA and protein vaccines suppresses the development of AIH

Because the flea salivary allergen, FSA1, has been identified and implicated as one of the causes of allergic dermatitis observed in cats and dogs (15), we cloned and expressed two regions of FSA1 encoding T cell epitopes at aa 100–114 and aa 66–80, designated
as pD100 and pD66, respectively (data not shown). We next used this AIH model to examine the ability of a co-inoculation strategy to protect animals from AIH following a flea allergen challenge. C57BL/6 mice were presensitized with various test immunogens and were then intradermally challenged with the flea extracts, or with histamine as a positive control or with PBS as a negative control 14 days after the second immunization (Fig. 2a). Naive mice served as a negative control group. AIH was inhibited only in the group coimmunized twice with the plasmid DNA pD100 plus total flea protein Ags (designated as pD100+F), whereas AIH was less inhibited by the construct pD66 plus total flea proteins (designated as pD66+F), which suggests that the pD100+F mix induced epitope-preferential suppression. To rule out that the observed inhibition was not due to a nonspecific factor such as a CpG motif within the plasmid backbone, AIH at the challenge site was monitored in the mice immunized with either the empty vector control plus total flea proteins (designated as Vector+F) or, alternatively, immunized with pD100 given as a prime injection with the flea protein administered as a boost (designated as pD100→F) (Fig. 2a). To further rule out the possibility that any other combination of DNA plus protein would induce a nonspecific inhibition, the mice were immunized either with pcD-VP1, encoding the VP1 protein of foot-and-mouth disease virus (9), plus flea proteins or with a chemically inactivated protein of foot-and-mouth disease virus plus pD100 (Fig. 2b) as additional controls. The results indicated that there was no inhibition of AIH by any of these combinations in controls, suggesting that the inhibition of AIH is indeed solely due to coimmunization with pD100+F, and further suggesting an Ag-specific type of inhibition.

Histological analysis revealed infiltrations by leukocytes and mast cells, and dermal edema and weakening of tissue structures in the skin lesions of mice immunized with total flea protein Ags (F) or Vector+F at the challenge sites (16–18); whereas, mice immunized with an active combination of pD100+F plus pD66 showed a normal intradermal structure that was free of inflammatory cells (Fig. 2c).

We next attempted to determine whether the inhibition of AIH induced by coimmunization was dose-dependent. For this purpose, the Ag-expressing plasmid pD100 at doses of 25, 50, 100, and 200 μg were coimmunized each with 100 μg of total flea proteins. The dosage of 50 μg of pD100 showed significant inhibition, which then reached maximal inhibition of AIH at 100 μg. A dose of 25 μg, however, exhibited only minimal effect on blocking lesion formation, as the animals developed rather severe reactions similar to those observed in animals inoculated with either total flea proteins (F), Vector+F (Fig. 2d), or with the positive control, histamine.

High levels of IL-4, IL-5, and IL-13 are characteristics of allergic reactions and these immune modulators are implicated in allergy severity (19–22). We next examined whether different profiles of these cytokines were associated with the co-inoculation regimens. Mice inoculated with total flea proteins or the co-inoculation of Vector+F produced higher level of expressed mRNA for IL-4, IL-5, and IL-13, whereas mice with the co-inoculation of pD100+F produced relatively low levels of these cytokines (Fig. 2e), suggesting that an anti-inflammatory immune regulatory function was obtained by the co-inoculation of pD100+F. No significant differences were found in the levels of IL-2 or IFN-γ among from splenocytes of mice immunized with pD100+F, Vector+F, total flea protein or naive control and adoptively transferred into syngeneic flea Ags sensitized mice. IDT challenges by flea Ags and measurements were performed 24 h after the adoptive transfer. *, p < 0.05; **, p < 0.01 compared with Vector+F group as indicated (n = 4 mice per group).

FIGURE 3. CD4+CD25+ T cells are responsible for the observed inhibitions. a, To test the regulatory function of T cells obtained from various immunizations in vitro, 5 × 10^6 of CD3+ T cells as the responder cells were isolated from the splenocytes of mice immunized with the flea Ags and added to 96-well plates. At the same time, 1 × 10^6 of lymphocytes as the regulatory cells from naive, total flea protein (F), Vector+F, and pD100+F immunized mice were also added to the same plate. Similarly, 1 × 10^6 non-T cells or T cells, purified CD8+ cells, CD4+ cells, or CD4+CD25+ T cells as the regulatory cells were purified from the spleens of mice immunized with Vector+F or pD100+F and added to plate. These cocultures were stimulated with flea Ag (50 μg/ml) in the presence of 1 × 10^6 bone marrow-derived DCs for 48 h in vitro. Proliferation was examined by MTS-PMS (Promega). Stimulation index (SI) shown in y-axis was determined by the formula: count of flea Ag-stimulated/count of nonstimulated cultures. b, To test the regulatory function of T cells obtained from various immunizations in vivo, 1 × 10^6 total T cells, purified CD8+, CD4+, 5 × 10^6 CD4+CD25−, and CD4+CD25+ T cells were isolated and were coimmunized each with 100 μg of total flea proteins. Inhibition was not due to a nonspecific factor such as a CpG motif, and the pD100+F mix induced epitope-preferential suppression. To rule out that the observed inhibition was not due to a nonspecific factor such as a CpG motif, the mice were challenged with the flea extracts, or with histamine as a positive control or with PBS as a negative control 14 days after the second immunization (Fig. 2a). Naive mice served as a negative control group. AIH was inhibited only in the group coimmunized twice with the plasmid DNA pD100 plus total flea protein Ags (designated as pD100+F), whereas AIH was less inhibited by the construct pD66 plus total flea proteins (designated as pD66+F), which suggests that the pD100+F mix induced epitope-preferential suppression. To rule out that the observed inhibition was not due to a nonspecific factor such as a CpG motif within the plasmid backbone, AIH at the challenge site was monitored in the mice immunized with either the empty vector control plus total flea proteins (designated as Vector+F) or, alternatively, immunized with pD100 given as a prime injection with the flea protein administered as a boost (designated as pD100→F) (Fig. 2a). To further rule out the possibility that any other combination of DNA plus protein would induce a nonspecific inhibition, the mice were immunized either with pcD-VP1, encoding the VP1 protein of foot-and-mouth disease virus (9), plus flea proteins or with a chemically inactivated protein of foot-and-mouth disease virus plus pD100 (Fig. 2b) as additional controls. The results indicated that there was no inhibition of AIH by any of these combinations in controls, suggesting that the inhibition of AIH is indeed solely due to coimmunization with pD100+F, and further suggesting an Ag-specific type of inhibition.

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Coimmunization of DNA and protein vaccines induces Ag-specific suppression. a, Schematic representation of adoptive transfer after the initial coimmunization by DNA, and flea Ags and T cell proliferations in vitro were tested after the transferred mice was simultaneously immunized with respective Ags. b, CD4+CD25− T cells at 1 × 10^6 were purified from lymphocytes of pD100+F and Vector+F immunized or naive mice and adoptively transferred into syngeneic naive mice. The mice were immunized with the specific flea Ags or nonspecific OVA Ag emulsified in CFA on the day of adoptive transfer. After 14 days, CD4+ T cells were isolated and their proliferations were performed in vitro with different stimulants as indicated. Results shown are representative of two independent experiments. *p < 0.05 compared with Vector+F transfers as indicated (n = 4 mice per group).

These groups, but IFN-γ and IL-2 was slightly higher in mice primed with pD100 and boosted by total flea proteins (Fig. 2e). This result again suggests that the induced inhibition of AIH by coimmunization was not likely due to the Th cell switching from Th2 to Th1 in the allergic responses.

Because flea Ag-triggered IgE- and IgG1-mediated allergic responses are well known and characterized (23, 24), we next investigated whether the construct pD100+F Ag could inhibit the induction of anti-flea IgE. The levels of anti-flea IgE and IgG1 in sera were therefore measured on days 14, 28, and 42 and were observed to reduce slightly in mice coimmunized with pD100+F compared with groups coimmunized with Vector+F or total flea proteins (p = 0.063) (Fig. 2f), further suggesting that the co-inoculation regimen did not directly influence the production of IgE and IgG1.

Proliferative CD4+ T cells are known to be involved in the development of AIH (25, 26). We next examined isolated CD4+ T cells from the spleen of mice coimmunized with total flea proteins, Vector+F, pD100+F, or pD100 primed and total flea protein boosted on day 14 after the second immunization to determine their ability to recall proliferative responses to flea Ags in vitro. Immunizations in the total flea protein, Vector+F, and prime-boost groups resulted in strong proliferative responses of CD4+ T cells, whereas the CD4+ T cells isolated from pD100+F-immunized mice showed little, if any, proliferation in response to the flea Ag stimulation (Fig. 2g). The result suggests that the inhibition of AIH observed by coimmunization of pD100+F is apparently related to the nonresponsiveness of Ag-specific CD4+ T cells. Furthermore, our results indicate that the coimmunization with pD100 and total flea protein induces an inhibition of the AIH via down-regulating the levels of inflammatory cytokines and the response of CD4+ cells. Collectively these data suggest that the inhibition of AIH is likely to be Ag-specific because the Ag-mismatched combinations did not produce any of the same effects as the coadministration of pD100+F (Fig. 2, a and b).

CD4+CD25− T cells are responsible for the suppression

We hypothesized that Ag-specific TREG cells have been induced by the coimmunization of flea DNA and flea protein vaccines. To test this hypothesis, we collected lymphocytes containing TREG cells from the coimmunized C57BL/6 mice and mixed them with the flea Ag-specific responder T cells of syngeneic mice that were previously immunized with flea Ags to examine their ability to inhibit recalled proliferative responses in vitro. As shown in Fig. 3a, lymphocytes from mice coimmunized with pD100+F significantly inhibited T responder cell-recalled immune responses. By contrast, lymphocytes from mice immunized with total flea proteins or Vector+F as well as lymphocytes from naive mice all failed to inhibit Ag-specific T responder cell proliferative responses (Fig. 3a, top left). This result indicates that TREG cells within the lymphocyte population that are likely generated during the coimmunization can suppress the Ag-specific T cell proliferative response. To further confirm this assertion, we purified total T cells or T cell subsets from the spleen of mice coimmunized with pD100+F and tested these cell sets individually for suppression of recall proliferation in a similar setting as described. Significant inhibition was observed from purified T cells, purified CD4+ cells, or purified CD4+CD25− cells from the mice coimmunized with pD100+F, but not from other subsets of cells in these mice (Fig. 3a, bottom left and right panels). However, the inhibition from CD4+CD25− cells is, in general, thought to be independent of any Ag sensitization, whereas the inhibition of CD4+CD25− cells in this reaction is seen to occur in an Ag-dependent manner (Fig. 3a, right panels).

We used an adoptive transfer procedure to further examine the TREG issue in vivo. Syngeneic recipient mice previously sensitized by flea Ags were adoptively transferred by receiving total T cells, CD4+ cells, CD4+CD25− cells, CD4+CD25− cells, or CD8+ cells isolated from C57BL/6 mice coimmunized with pD100+F, total flea proteins, or Vector+F, respectively. We next performed
FIGURE 5. CD4+CD25+ T_{Reg} cells from the coimmunization express both IL-10 and IFN-γ. After the second coimmunization, CD4+CD25+ T cells were purified by depletion of CD4+CD25+ cells from CD4+ cells from the spleen of mice immunized by total flea protein (F), Vector+F, and pD100+F on days 1, 3, 7, and 14 and analyzed for their intracellular cytokine productions of IL-10 (a), IFN-γ (b), IL-4 (c), top panels, and FoxP3 (d) left panel, by flow cytometry. Data depicting mean and SEM of the percentage of IL-10 (a), IFN-γ (b), IL-4 (c), bottom panels, and FoxP3 (d), right panel, were shown. 

* e, IL-10 and Foxp3 coexpression. Total lymphocytes of mice after the second coimmunization were stained with anti-CD4-FITC and anti-CD25-PE Cy5 mAb, and then intracellular stained with anti-IL-10-allophycocyanin and anti-Foxp3-PE mAb. Both CD4+CD25+ (R1) and CD4+CD25- (R2) T cells were gated for the coexpression of IL-10 and Foxp3. Results shown are representative of three experiments. Percentages represent percent of double positive cells. * p < 0.05 compared with Vector+F group as indicated (n = 4 mice per group).
IDT 24 h after the transfer on all recipient mice. Total T cells and CD4+ and CD4+CD25+ T cells from mice immunized with the pD100+F, but not from mice coimmunized with total flea protein or Vector+F, were all able to suppress the development of AIH (Fig. 3b). In contrast, CD8+ T cells isolated from all three immunization groups and cells from the naive control mice did not suppress this allergic reaction. Both in vitro and in vivo results were consistent and indicated that CD4+CD25+ TREG cells can mediate the suppression of AIH. Both flea-allergic or flea allergen-protected mice harbored CD4+CD25+ cells that inhibited the allergic responses to intradermal flea allergen injections in the sensitized recipient mice. In contrast, only flea allergen-protected donor mice gave CD4+CD25− cells that inhibited the responses to flea allergen in sensitized recipient mice.

**CD4+CD25+ T cells from the coadministration procedure mediate Ag-specific suppression**

To investigate whether the CD4+CD25+ TREG cells play a role in an Ag-specific inhibition, we adoptively transferred these cells taken from C57BL/6 mice previously coimmunized with pD100+F into syngeneic recipient mice. The recipient mice were previously immunized twice at a biweekly interval using flea Ags or an OVA Ag emulsified in CFA. On day 14 after the last immunization, T cells were isolated and tested for their ability to proliferate with either flea Ag or OVA in vitro. T cells from the recipient mice that were subsequently immunized with the flea Ags did not respond to the flea Ag-induced stimulation in vitro; whereas, the T cells from recipient mice subsequently immunized with OVA Ag emulsified in CFA responded well to OVA stimulation, but not to the flea Ag stimulation in vitro. As the controls, the naive mice immunized with flea Ag respond well to the flea Ag stimulation, but not to the OVA stimulation in vitro and vice versa (Fig. 4). This finding indicates that adoptively transferred CD4+CD25+ TREG cells only inhibit the flea Ag-specific T cell priming and proliferation, whereas the responses from the irrelevant Ag-specific T cells were not affected.

Taken together, these data demonstrate that CD4+CD25+ TREG cells were induced by the procedure of coimmunizing plasmid DNA expressing flea protein and flea peptide vaccines. These results appear to be due to unique CD4+ TREG cells as they function in an Ag-specific manner.

**The characterization of TREG cells from the coimmunization**

To determine whether TREG cells induced by co-inoculation express certain types of well-documented cytokines and unique markers associated with TREG cells (27–29), CD4+CD25− cells were isolated from the mice immunized with total flea protein, Vector+F, or pD100+F on days 1, 3, 7, and 14 postimmunization. T cell profiles were analyzed by FACS using intracellular staining with specific fluorescently labeled Abs. Over the course of 14 days, the CD4+CD25− T cells isolated from mice coimmunized with pD100+F were seen to induce increased populations producing IL-10 (Fig. 5a), IFN-γ (Fig. 5b), and Foxp3 (Fig. 5d) and a minimal amount of IL-4 (Fig. 5c). In contrast, for the mice immunized with Vector+F or total flea protein (data not shown), we observed that more CD4+CD25− T cells producing IL-4, but less T cells producing Foxp3, IL-10, or IFN-γ. Because the transcriptional factor Foxp3 has been demonstrated to be a hallmark of the TREG cells (30, 31), the coinmunization-induced CD4+CD25− TREG cells can be thus categorized into the regulatory class of T cells with a unique phenotype. To examine both IL-10 and Foxp3 coexpression in the same cell to ascertain the TREG cells being a homogenous population of the same profile, both CD4+CD25− and CD4+CD25+ T cells from lymphocytes of mice after the second coimmunization were double stained and subsequently stained with anti-IL-10-allophycocyanin and anti-Foxp3-PE mAbs intracellularly. The results showed that the co-inoculation group pD100+F induced more CD4+CD25− T cells expressing higher levels of IL-10-Foxp3+ TREG cells compared with other groups (Fig. 5e). In contrast, as a positive control, CD4+CD25+ T cells from each group expressed both Foxp3 and IL-10.

The size of populations expressing T cell activation markers for CD44 and CD69 found low for CD62L were found equally high among the immunized groups (Fig. 6a), suggesting that the induced CD4+CD25− TREG cells were fully activated by the coimmunization procedure.

To analyze the T helper phenotype for the induced CD4+CD25− TREG cells based on the observed cytokine expression patterns we described, the expression of both T-bet and GATA3 genes of the CD4+CD25− T cells derived from mice immunized with total flea protein, Vector+F, or pD100+F by the RT-PCR method was determined. The results show that the CD4+CD25− T cells from the pD100+F or pD100-immunized mice, but not from the total flea protein- or Vector+F-immunized mice, expressed a higher level of T-bet and GATA3 genes of the CD4+CD25− T cells derived from mice immunized with total flea protein, Vector+F, or pD100+F by the RT-PCR method was determined. The results show that the CD4+CD25− T cells from the pD100+F or pD100-immunized mice, but not from the total flea protein- or Vector+F-immunized mice, expressed a higher level of T-bet and GATA3, which is a characteristic of Th2 cells (34, 35). These results indicate that coinmunization of pD100+F elicits the Th1 response, which is the same as the observed for the group immunized by pD100, suggesting that factors such as IFN-γ play an influential role in the development of naive T to Th1 cell transition. This concept is partly supported by the observation of elevated IFN-γ production elicited from the CD4+CD25− TREG cells as shown in Fig. 5b.

Collectively, these data demonstrate that CD4+CD25− TREG cells induced by the coinmunization of cognate DNA and protein vaccines engender adaptive Th1 phenotypic TREG cells that are
able to suppress the Ag-specific CD4\(^+\) T cell proliferative function and the specific allergic response.

**Analysis of maturation and presentation of DCs from the coimmunized mice**

Because APCs activate T cells to promote adaptive immunity, we reasoned that the induction of CD4\(^+\)CD25\(^-\) T\(_{\text{REG}}\) cells via specific APC activation was induced by the co-inoculation of cognate DNA plus protein vaccines. To address this question, we examined phenotypes and functions of DCs and their influence on naive T cells. First, we analyzed the effects of coimmunization on maturation of DC. As depicted in Fig. 7, DCs isolated from the spleen of mice 48 h after pD100+F immunization expressed high levels of CD80, CD86, MHC class II (Fig. 7a) and more IL-10, but similarly levels of IL-12, IL-6, IL-1\(\alpha\), IFN-\(\alpha\), IFN-\(\beta\), and TNF-\(\alpha\) compared with levels from total flea protein or Vector+F groups (Fig. 7b), which are characteristics of matured DCs. The expression of these same markers on the immature DCs from naive control mice remained at relatively low levels. The results suggest that the flea Ag coimmunization procedure enables the immature DCs to be induced and become matured. However, we observed that the level of CD40 expression was dramatically reduced in the pD100+F-immunized mice compared with other immunized groups (Fig. 7a), suggesting that a unique phenotype of DC was involved in the observed induction of these Ag-specific T\(_{\text{REG}}\) cells.

Secondly, to assess the DC function, DCs isolated from mice coimmunized with Vector+F or total flea protein were observed to have the ability to activate allogeneic T cells to proliferate, whereas as expected, the immature DCs of naive mice have no such ability (Fig. 7c). Interestingly, DCs from mice coimmunized with pD100+F had a restricted capacity to activate allogeneic T cells to proliferate (Fig. 7c), suggesting that an alternative mechanism of T\(_{\text{REG}}\) induction is being induced in the coimmunization group.

**DCs from the coimmunized mice convert naive T cells into T\(_{\text{REG}}\) cells in vitro**

To explore whether the DCs of coimmunized mice are able to convert naive T cells to a T\(_{\text{REG}}\) phenotype, we cocultured DCs from pD100+F mice coimmunized with syngeneic naive CD4\(^+\) T cells in vitro, and subsequently characterized the resulting CD4\(^+\) T cells by FACS on days 1, 3, and 7 (Fig. 8a). We observed that these T cells up-regulated higher levels of CD44 and CD69, but lower levels of CD62L (data not shown), suggesting again that the DCs have the capacity to activate naive T cells. Similar results were obtained from analysis of DCs from mice coimmunized with Vector+F or total flea protein. Furthermore, we found that the activated DCs from the pD100+F-immunized group induced more T cells to produce IL-10 but fewer cells to produce IL-4 (Fig. 8b), and IFN-\(\gamma\)-producing cells were not significantly increased.
However, DC cells from Vector+F- or total flea protein-immunized groups induced more T cells expressing IL-4, but fewer T cells expressing IL-10 and IFN-γ (Fig. 8b). Cytokine production was analyzed after three rounds of restimulation with fresh isolated DCs from the pD100+F-immunized mice. These studies demonstrated an increase in the production of IL-10 in DCs from pD100+F-immunized mice, which is one of the characteristics of T_{REG} cells, and an increase in IFN-γ, which supports the previous results of elevated IFN-γ depicted in Fig. 5b. To further characterize the function of the in vitro-induced T_{REG} cells, a standard allogeneic MLR was used. The responder T cells were stimulated by allogeneic DCs, and the syngeneic T_{REG} cells were subjected to MLR to see whether they block the proliferation of responder T cells. The data show that the proliferation of responder T cells was inhibited by the presence of IL-10+CD4+ T cells, but not by T cells isolated from cocultures with DCs of mice coimmunized with Vector+F and total flea protein or from the control animals (Fig. 8c). These data demonstrate that DCs from the pD100+F-immunized mice can convert naive T cells into T_{REG} cells in vitro.

DCs from the coimmunized mice convert naive T cells into T_{REG} cells in vivo

To validate the conversion into T_{REG} cells in vivo, we cotransferred DCs collected from BALB/c mice after coimmunization of the pD100+F group with syngeneic naive CD4+ T cells into nude mice (nu/nu) and analyzed the transferred T cells on days 3 and 7 by FACS for intracellular expression of cytokines and T_{REG} markers (Fig. 9a). We observed that most cotransferred T cells expressed IL-10 (Fig. 9b), IFN-γ (Fig. 9c), and FoxP3 (Fig. 9d), but not IL-4 (Fig. 9e). However, most cotransferred T cells with DCs from mice coimmunized with pD100+F expressed IL-4, but not IL-10 and IFN-γ, supporting the in vitro conversion data. Production of IL-10 was seen to be elevated after two rounds of restimulation with the DCs freshly isolated from the pD100+F coimmunized mice. These data further demonstrate that DCs from mice coimmunized with pD100+F can convert naive T cells into T_{REG} cells in vivo. Consistent with our previous results, such conversion was only
made within the CD4⁺CD25⁺ populations because the frequency of CD25⁺ was not observed to be changed at all (Fig. 9).

Discussion
Our studies demonstrated that the co-inoculation of flea Ag-expressing DNA vaccine with the flea protein vaccine induces adaptive TREG cells, which inhibited the AIH induced by flea allergen challenge in vivo. These TREG cells exhibit a phenotype of CD4⁺CD25⁺Foxp3⁺, express IL-10, IFN-γ, and T-bet, and suppress Ag-specific immune responses in vivo and in vitro. We have identified important cellular components in the induction of TREG cells in this system because MHC class II⁺CD40low DC populations were induced by such coimmunization and, in turn, convert the naive T cells into TREG cells.

It has recently been demonstrated and frequently observed that naturally occurring TREG cells can inhibit autoimmune diseases (6, 7, 36) and allergic responses (4, 5, 37), as well as induce tolerance (38), affect antitumor immunity (39), and regulate the activation of...
other peripheral T cells. These types of T\textsubscript{REG} cells have the phenotype CD4\textsuperscript{+}CD25\textsuperscript{+} and constitute ~10% of peripheral CD4\textsuperscript{+} T cells in normal mice. Several studies have demonstrated that some peripheral CD4\textsuperscript{+} and CD25\textsuperscript{+} T cell populations could be also induced to show regulatory capabilities, including T\textsubscript{reg} cells (28) and Th3 cells (8). T\textsubscript{reg} cells are defined by their ability to produce large amounts of IL-10, with or without TGF-\(\beta\); however, Th3 cells produce mainly TGF-\(\beta\) (40). Interestingly, one subpopulation of natural CD8\textsuperscript{+} T cells was reported to have regulatory properties (41). Differing from these findings, our study with coimmunization of flea Ag DNA and protein demonstrated the induction of a new phenotypic type of T\textsubscript{REG} cells producing not only IL-10 (Fig. 5a) but also IFN-\(\gamma\) (Fig. 5b) that suppressed the development of allergic reactions in a specific manner. In addition, the expression of T-bet (Fig. 6c) suggested that the T\textsubscript{REG} cells are related to Th1 cells.

Although DCs were most frequently demonstrated to be related with the induction of protective immunity, recent results show that DCs also play a key role in the development of T\textsubscript{REG} cells, including natural CD4\textsuperscript{+}CD25\textsuperscript{+} T\textsubscript{REG} (42), Th3 (43), and adaptive T\textsubscript{reg} (44, 45) cells. The costimulatory signals such as the ICOS-ICOS ligand (46), B7-CTLA-4 (47), and CD40-CD40L (48) have been implicated to play key roles in inducing T\textsubscript{REG} cells from DCs.

Adaptive T\textsubscript{reg} cells have been observed to be induced by DCs processing a suboptimal immunogen or subimmunogen to inhibit normal T cell function via sequestration of IL-10, TGF-\(\beta\), or both (49). It has been further demonstrated that immature DCs can drive the differentiation of T cells to produce IL-10, TGF-\(\beta\), and IFN-\(\gamma\), but not the production of IL-4 and IL-2. Following this differentiation, T cells are prone to be hyporesponsive to Ag-specific and polyclonal activation (50). In addition, evidence has shown that suboptimal activation of DC by minute Ag stimulation can induce T\textsubscript{reg} conversion. This stimulation of DCs arises due to the lack of costimulation signaling and thus affects a tolerance signal to T cells (51). Whether the coimmunization of cognate DNA and protein vaccines is presenting a “subimmunogenic” response to DCs, which subsequently drives T\textsubscript{reg} conversion remains unknown. The coimmunization of DNA and protein vaccines in animals induces a subtle difference on the DCs with the observed MHC class II\textsuperscript{+}, CD80\textsuperscript{high}, CH50\textsuperscript{high}, and CD40\textsuperscript{low} phenotypes (Fig. 7a). Whether such a difference makes the DCs suboptimal and leads to the subsequent conversion of the Ag-specific T\textsubscript{REG} cells from naive T cells is still an unanswered question and requires further investigation. Understanding the complexity of signal pathways in DCs after this type of coimmunization may reveal an important immune regulatory mechanism in the immune system.

It has been well documented that the development of allergic diseases, including dermatitis and asthma, is mainly mediated by the activation and function of Th2 cells (18, 52). Therefore, several studies reported that the Th1 type of response could induce protective immunity against asthma (53, 54) or allergy (3) by inhibiting the function of the Th2 cells. However, other studies showed that Th1 cells could not successfully suppress the Th2 cell-mediated inflammation, but instead seemed to potentiate the infiltration of inflammation-mediated cells or inflammatory responses (55, 56). Furthermore recent studies showed that, in some cases, the development of inflammation and autoimmune diseases were directly related to a new type of Th17 Subpopulation (57, 58), but not to the Th2 or Th1 cells, all of which complicates the current understanding. No matter what the situation, these studies suggest that there are some other forms of response that regulate the development of inflammation. In this study, we demonstrated that a new subtype of regulatory cell belonging not to Th1, but expressing both IL-10 and IFN-\(\gamma\), is induced by coimmunization of DNA and protein and can successfully suppress the development of hyperallergic reactions in mice.

Recently, Inoue and Aramaki (59) demonstrated that transfer vaginal administration of CpG oligodeoxynucleotides may shift the immune response from type Th2 to Th1 in NC/Nga mice, which spontaneously develop atopic dermatitis-like symptoms and high Th2 immune responses. They also found that CpG oligodeoxynucleotide application could induce CD4\textsuperscript{+}CD25\textsuperscript{+} T\textsubscript{REG} cells to appear in the skin lesions. Different to their finding, our results in this study demonstrated that coimmunization with flea Ag pD100 and total flea protein vaccines produced an Ag-specific expansion of CD4\textsuperscript{+}CD25\textsuperscript{+} IL-10\textsuperscript{+}Foxp3\textsuperscript{+} IFN-\(\gamma\)\textsuperscript{+} T cells, which then inhibited flea allergy dermatitis in mice model. In contrast, directing the immune system toward the Th1 response cannot suppress the flea allergy dermatitis in mice. Thus, we concluded that the coimmunization procedure induced an Ag-specific immunosuppression rather than the global immune effects mediated by the Th1/Th2 biased response. Furthermore, the suppression induced by the coimmunization seems to be epitope-specific because the less inhibition induced by the epitope 66 coimmunization (pD66+F) was exhibited (Fig. 2a). The reason for epitope specificity is not fully understood and required further investigation.

The coexpression of IL-10 and IFN-\(\gamma\) findings were somewhat ambivalent because IL-10 has been considered to be an suppressive cytokine (60–63) known to inhibit Th1 cytokine expression (64). However, recently, the Umetsu group (29) has also shown that the induction of a previously unknown adaptive T\textsubscript{REG} cell expresses both IL-10 and IFN-\(\gamma\) by CD8\textsuperscript{+} DCs with an ability to protect mice against airway hyperreactivity. More interestingly, the receptors of both IFN-\(\gamma\) and IL-10 belong to the class II cytokine receptor type (65, 66), and the three-dimensional structures of these two cytokines shared some degree of similarity with their pattern of \(\alpha\)-helical structures (67). Thus, the identification of T\textsubscript{REG} cells by their cytokine expression patterns or other specific cell-type genes are still a very complicated and evolving issue.

These studies, nonetheless, define for the first time to our knowledge an important and unique immune regulatory process and provide a model for further investigations of its signaling cascades. Ultimately, exploitation of these unique cellular immune regulatory pathways and manipulation of their induction may reveal important mechanisms that will lead to the development of novel therapeutic approaches against allergic and other related immune disorders in both humans and animals.

Acknowledgments
We express appreciation to Dr. David B. Weiner and Dr. Richard X. Ascierno for critical review and valuable suggestions. We thank Dr. Jane Q.L. Yu, Zhonghua He, and Qinghong Zhu for assistance in this work.

Disclosures
T. Ng and H.J. Chu are employees of, B. Wang is a consultant of Fort Dodge Animal Health, a Wyeth company. Contents of this publication are subject of patent application filing.

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