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## TLR2 Agonists Enhance CD8<sup>+</sup>Foxp3<sup>+</sup> Regulatory T Cells and Suppress Th2 Immune Responses during Allergen Immunotherapy

Yi-Giien Tsai, Kuender D. Yang, Dau-Ming Niu, Jien-Wen Chien and Ching-Yuang Lin

*J Immunol* 2010; 184:7229-7237; Prepublished online 7 May 2010;

doi: 10.4049/jimmunol.1000083

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# TLR2 Agonists Enhance CD8<sup>+</sup>Foxp3<sup>+</sup> Regulatory T Cells and Suppress Th2 Immune Responses during Allergen Immunotherapy

Yi-Giien Tsai,<sup>\*,†</sup> Kuender D. Yang,<sup>‡</sup> Dau-Ming Niu,<sup>§</sup> Jien-Wen Chien,<sup>\*,†</sup> and Ching-Yuang Lin<sup>¶</sup>

Pam3CSK4, a synthetic TLR2 ligand, has been shown to expand CD4<sup>+</sup> regulatory T cells (Treg cells). Less is known about the function of CD8<sup>+</sup> Treg cells than about the function of CD4<sup>+</sup> Treg cells generated during allergen-specific immunotherapy (IT). This study investigated whether *Dermatophagoides pteronyssinus*-specific IT could expand the CD8<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg population and whether Pam3CSK4 could enhance the Treg population. PBMCs were isolated from healthy control subjects and from mite-sensitive asthmatic patients during IT at three specific times: before IT and 6 mo and 1 y after the maximum-tolerated dose. This study was performed without a placebo-controlled group. *D. pteronyssinus*-specific IT induced a significant increase in CD8<sup>+</sup>Foxp3<sup>+</sup> Treg cells expressing intracellular IL-10 and granzyme B. Costimulation of PBMCs with Pam3CSK4 and *D. pteronyssinus* 2 expanded the CD8<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg population and inhibited *D. pteronyssinus* 2-induced IL-4 production. Pam3CSK4-treated CD8<sup>+</sup>CD25<sup>+</sup> Treg cells directly suppressed CD4<sup>+</sup> T cell proliferation by cell-contact inhibition. TUNEL revealed that CD8<sup>+</sup>CD25<sup>+</sup> Treg cells, but not CD4<sup>+</sup>CD25<sup>+</sup> Treg cells, directly induced CD4<sup>+</sup>CD45RO<sup>hi</sup> apoptosis. Our results provide direct evidence that Pam3CSK4 induces an immunomodulatory effect by inducing CD8<sup>+</sup> Treg cells; therefore, it may be a good adjuvant for the treatment of mite allergies. *The Journal of Immunology*, 2010, 184: 7229–7237.

Toll-like receptors play an important role in bridging innate and adaptive immune responses in the development of pathogen-associated allergic diseases (1, 2). Studies showed that TLR2 agonists protect against allergy and asthma by modulating the immune response Th1/Th2 balance (2–4). Recent studies suggested that TLR2 directly enhances CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cell (Treg cell) proliferation and function through Foxp3 expression (5, 6), a mechanism that may be beneficial for the treatment of allergic disorders (7).

CD8<sup>+</sup> Treg cells' involvement in maintaining self-tolerance was recently identified (8, 9). These cells' surface markers include CD25, CD103, and CD122 (10, 11). CD8<sup>+</sup> Treg cells with regulatory function express transcription factor Foxp3 (11–13). Human CD8<sup>+</sup> Treg cells are implicated in various infectious diseases (14–16) and autoimmune disorders, including multiple sclerosis (17) and inflammatory bowel disease (18). In the tumor microenvi-

ronment, CD8<sup>+</sup>CD25<sup>+</sup> Treg cells have a suppressive ability that typically is associated with CD4<sup>+</sup> Treg cells (19–21).

Unlike CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells generated in the thymus, the suppressive CD8<sup>+</sup>Foxp3<sup>+</sup> Treg cells appear after primary Ag stimulation, suggesting that they are amplified by TCR stimulation (22). CD8<sup>+</sup> Treg cells can suppress cellular proliferation of CD4<sup>+</sup> naive and effector T cells via cell–cell contact lysis or soluble factors, such as IL-10 and TGF- $\beta$  (23). Cottalorda et al. (24) demonstrated that TLR2 engagement on CD8<sup>+</sup> cells induced a sustained expression of CD25, with an increase in Treg function. However, it is not clear whether CD8<sup>+</sup> Treg cells have detrimental effects on immune tolerance from allergic diseases.

Allergen-specific immunotherapy (IT) by repeated s.c. administration of increased doses of allergen extracts has a long-lasting effect on immune tolerance to common environmental allergens (25–27). Recent studies suggested that the induction of CD4<sup>+</sup> Treg cells might be associated with suppression of allergic responses in patients after successful IT (25). It was demonstrated that the TLR2 synthetic agonist Pam3CSK4 has therapeutic potential to decrease the mite allergen-induced Th2 immune response (28, 29); thus, it may be useful as an adjuvant in immunotherapy for allergic disease (30). Our study investigated whether *Dermatophagoides pteronyssinus*-specific IT can enhance CD8<sup>+</sup> Treg populations, as well as whether Pam3CSK4 increases CD8<sup>+</sup>Foxp3<sup>+</sup> Treg cells and may help to suppress a mite allergen-induced Th2 immune response. Findings may yield further evidence and elucidate a mechanism for novel immunotherapeutic prevention and treatment.

## Materials and Methods

### Subjects

Fifty children with mild intermittent to moderately persistent asthma and with sensitivity to house dust mites (*D. pteronyssinus*), demonstrated by a positive skin-prick test ( $\geq 2+$ ) and an IgE-specific test greater than third grade (specific IgE level  $>3.5$  kU/l) using the CAP system (Pharmacia

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Received for publication January 26, 2010. Accepted for publication April 2, 2010.

This work was supported in part by grants from the Changhua Christian Hospital and National Science Council, Taiwan, ROC (NSC 98-2314-B-371-001-MY2) and China Medical University (CMU96-217).

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Abbreviations used in this paper: FEV1, forced expiratory volume in 1 s; IT, immunotherapy; PI, propidium iodide; Treg cell, regulatory T cell.

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Diagnostics, Uppsala, Sweden), were enrolled in this study and received *D. pteronyssinus*-specific IT. The patients received the maximum monthly tolerated dose, according to a previously described standardized protocol, and were followed for 1 y (27). Patients were instructed to record asthmatic scores (27), and pulmonary function forced expiratory volume in 1 s (FEV1) (Sensomedics, Yorba Linda, CA) was measured before and after IT. Fifty children with normal serum IgE levels and who were negative for skin-prick tests were selected as controls. The subjects ranged in age from 5–17 y. All patients completed the study. The study was performed with the approval of the institutional review board, and parents of all subjects provided written informed consent.

### Abs and reagents

Synthetic lipopeptide Pam3CSK4 (InvivoGen, San Diego, CA) for TLR2 ligand was prepared in sterile PBS. Recombinant *D. pteronyssinus* 2 (Lot 2836, Indoor Biotechnologies, Cardiff, U.K.) was used as the allergen. Anti-human CD3, CD4, CD8, CD25, CD45RO, Foxp3, granzyme B, and IL-10 mAbs and isotype-matched control mAbs conjugated with FITC, PE, Electron Coupled Dye, and PC5 (anti-human IgG1 PC5-conjugated mAb for CD8 and anti-human IgG1 PE-conjugated mAb for Foxp3) were obtained from BD Biosciences (San Jose, CA).

### Cell isolation and cell culture

PBMCs were isolated by Ficoll-Paque gradient centrifugation (Pharmacia Biotech). A total of  $1 \times 10^6$  cells were cultured with recombinant *D. pteronyssinus* 2 (10 µg/ml) or Pam3CSK4 (5 µg/ml) for 5 d and divided on 96-well culture plates in RPMI 1640 culture medium supplemented with L-glutamine (2 mmol/l), HEPES (20 mmol/l), sodium pyruvate (91 mmol/l), streptomycin (50 ng/ml), penicillin (100 IU/ml), and 10% FBS (Bio-Whittaker, Walkersville, MD). In some experiments, CD8<sup>+</sup> or CD4<sup>+</sup> cells were depleted directly from PBMCs using microbeads, according to manufacturer's protocol (BD Biosciences). CD8<sup>+</sup>CD25<sup>+</sup> T cells were isolated using a CD8<sup>+</sup> T cells enrichment kit, followed by separation with CD25 microbeads (BD Biosciences). The purity of the CD8<sup>+</sup>CD25<sup>+</sup> T cell population analyzed by flow cytometry was >95%. CD4<sup>+</sup>CD25<sup>hi</sup> Treg cells from normal subjects were purified using the EPICS ALTRA high-speed cell sorter (Beckman Coulter, Fullerton, CA) and were used as positive controls for intracellular Foxp3 expression.

### Protein extraction and Western blot analysis

Twenty milliliters of peripheral blood was drawn from patients; isolated PBMCs were cultured for 5 d with *D. pteronyssinus* 2 stimulation and then sorted for purified CD8<sup>+</sup>CD25<sup>+</sup> Treg cells ( $5 \times 10^5$ ) for Western blotting. Purified CD8<sup>+</sup>CD25<sup>+</sup> Treg cellular protein was extracted by cell-lysis buffer (Roche, Basel, Switzerland). Cytoplasm proteins and nuclear proteins were obtained by NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL). We determined the protein levels of nuclear Foxp3 by Western blot analysis. Equal amounts of (CD8<sup>+</sup>CD25<sup>+</sup> T cells) proteins in each study group were determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). The cellular proteins were resolved by 10% SDS-PAGE. After electrophoresis, proteins were transferred onto a nitrocellulose membrane, blocked with 5% nonfat milk in PBS/Tween 20 (0.1%), and probed with Foxp3 Ab (Abcam, Cambridge, U.K.). Following incubation with primary Abs for 1 h, the membrane was washed and incubated with HRP-conjugated anti-mouse or anti-rabbit IgG Ab (1:10,000 in PBS/Tween and 1% BSA) and visualized using an ECL system (Pierce).

### ELISA

PBMCs were stimulated with *D. pteronyssinus* 2 (10 µg/ml) in the presence or absence of Pam3CSK4 (5 µg/ml) for 5 d, and their supernatants were evaluated for IL-4 and -10 and IFN-γ content by ELISA (R&D Systems, London, U.K.).

### Flow cytometry

Cells were fixed with 4% paraformaldehyde and washed with PBS containing 0.2% BSA. After washing, cells were stained for 30 min with fluorescein-conjugated mAbs. CD8<sup>+</sup>CD25<sup>+</sup> Treg cells were permeabilized and then stained with PE-conjugated, anti-Foxp3 mAb (BD Biosciences). For intracellular IL-10 and granzyme B cytokine staining, PBMCs were activated with PMA (10 ng/ml) and ionomycin (1 µg/ml) for the last 5 h of incubation, and brefeldin A (10 µg/ml; Sigma-Aldrich, St. Louis, MO) was added for the final hours of stimulation. Cells were fixed, permeabilized, stained using standard procedures (eBioscience, San Diego, CA), and analyzed using a FACScan flow cytometer (FC500, Beckman Coulter), acquiring 10,000 events.

### Cell proliferation assay and Transwell experiments

PBMCs and CD8<sup>+</sup>-depleted PBMCs were labeled with 5 µM CFSE (Invitrogen, Carlsbad, CA) for 15 min at 37°C. Cells were washed twice and stimulated with anti-human CD3 mAb (1 µg/ml; positive control) or Pam3CSK4 (5 µg/ml) for 5 d. For the CFSE-suppression assay (8), CD8<sup>+</sup>CD25<sup>+</sup> Treg cells were added to the culture autologous, CD8-depleted, and CFSE-labeled PBMCs at a 1:10 ratio, and CD4<sup>+</sup> T cell proliferation was analyzed by flow cytometry. Transwell experiments were carried out in 24-well plates (0.4 µm pore size, Nunc, Roskilde, Denmark). The CFSE-labeled CD8<sup>+</sup>-depleted PBMCs and CD8<sup>+</sup>CD25<sup>+</sup> Treg cells were placed in the upper chamber, and the CFSE-labeled CD8<sup>+</sup>-depleted PBMCs were placed in the lower chamber. The culture supernatant of CD8<sup>+</sup>CD25<sup>+</sup> Treg cells and PBMCs was added to CFSE-labeled CD8<sup>+</sup>-depleted PBMCs culture to confirm the inhibitory effect of soluble factors. After culturing, the proliferation of CD4<sup>+</sup> T cells was assessed by CFSE fluorescence with flow cytometric analysis.

### Detection of CD4<sup>+</sup>CD45RO<sup>+</sup> T cell apoptosis

To assess whether the presence of CD4<sup>+</sup> or CD8<sup>+</sup> Treg cells affects the apoptosis of CD4<sup>+</sup>CD45RO<sup>+</sup> T cells during IT, purified CD4<sup>+</sup>CD25<sup>+</sup> or CD8<sup>+</sup>CD25<sup>+</sup> Treg cells were added to autologous CD25<sup>+</sup>-depleted PBMCs with *D. pteronyssinus* 2 stimulation for 5 d. The apoptosis rate of CD4<sup>+</sup>CD45RO<sup>+</sup> T cells was obtained by flow cytometry after labeling DNA strand breaks using a TUNEL kit (Mebstain Kit, Immunotech, Luminy, France), as mentioned above (27). To confirm TUNEL data, we used an Annexin V-propidium iodide (PI)-labeling kit (BD Biosciences), followed by flow cytometry, to measure apoptosis.

### Statistical analysis

All data presented are mean  $\pm$  SD. Differences between the means before and after IT were analyzed using the paired Student *t* test. Differences of means compared with each treatment group were analyzed using ANOVA, followed by the Duncan test. A *p* value <0.05 was considered significant.

## Results

### Increase in CD8<sup>+</sup> Treg cells after IT

All asthmatic subjects who received *D. pteronyssinus* IT had improved asthmatic scores and increased pulmonary function (FEV1) after 1 y of treatment (*p* < 0.05) (Tables I, II).

Foxp3 is the essential transcription factor for the suppressor function of Treg cells. To study whether CD8<sup>+</sup> Treg cells were induced by specific *D. pteronyssinus* IT, Treg cells were analyzed by flow cytometry for surface markers and intracellular Foxp3 expression simultaneously. CD8<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells increased after IT of asthmatic subjects (Fig. 1A). The number of CD8<sup>+</sup>Foxp3<sup>+</sup> cells in *D. pteronyssinus* 2-stimulated PBMCs had increased after 6 mo and 1 year of IT ( $4.35\% \pm 2.38\%$  versus  $10.75\% \pm 3.14\%$  and  $11.30\% \pm 2.65\%$ ; before IT versus after 6 and 12 mo of IT, respectively) (Fig. 1B). Purified CD8<sup>+</sup>CD25<sup>+</sup> T cells isolated after *D. pteronyssinus* 2-stimulated PBMCs were analyzed by flow cytometry, and representative results are shown (Fig. 1C). Increased expression of Foxp3 by purified CD8<sup>+</sup>CD25<sup>+</sup> T cells by Western blot analysis was observed during IT (Fig. 1D), confirming the flow cytometry results.

### *D. pteronyssinus*-specific IT increased CD8<sup>+</sup>CD25<sup>+</sup> Treg cells expressing granzyme B and IL-10

Experiments were performed to investigate whether the increase in CD8<sup>+</sup>CD25<sup>+</sup> Treg cells was associated with IL-10 and granzyme B expression. *D. pteronyssinus* 2-stimulated PBMCs were

Table I. Patient characteristics

	IT Group	Control Group
Patients ( <i>n</i> )	50	50
Age (y; mean $\pm$ SD)	12.15 $\pm$ 3.56	12.06 $\pm$ 3.71
Gender (male/female)	26:24	30:20
Skin prick test (grade)	3.7 $\pm$ 0.59	–

Table II. Clinical response, including symptoms score, FEV1, and *D. pteronyssinus*-specific IgE changes after IT

	Baseline (Mean ± SD)	Post IT (Mean ± SD)
<i>D. pteronyssinus</i> -specific IgE (kU/l)	75.71 ± 15.47	43.96 ± 13.63*
Asthma score	3.4 ± 0.5	0.8 ± 0.7*
FEV1 (%)	78.56 ± 8.43	91.16 ± 9.12*

\**p* < 0.05, compared with baseline.

activated for an additional 4 h with PMA and ionomycin and then stained for intracellular IL-10 and granzyme B to characterize their expression in CD8<sup>+</sup>CD25<sup>+</sup> T cells (Fig. 2A). PBMCs from asthmatic patients contained greater numbers of CD8<sup>+</sup>CD25<sup>+</sup>IL-10<sup>+</sup> T cells after 1 y of IT than before IT (12.69% ± 3.39% versus 5.58% ± 3.16%, respectively; *p* < 0.05) (Fig. 2B) as well as greater numbers of granzyme B-expressing CD8<sup>+</sup>CD25<sup>+</sup> T cells (13.59% ± 3.85% versus 6.22% ± 3.10%, respectively; *p* < 0.05) (Fig. 2C). This suggests that CD8<sup>+</sup> Treg cells contribute to successful treatment.

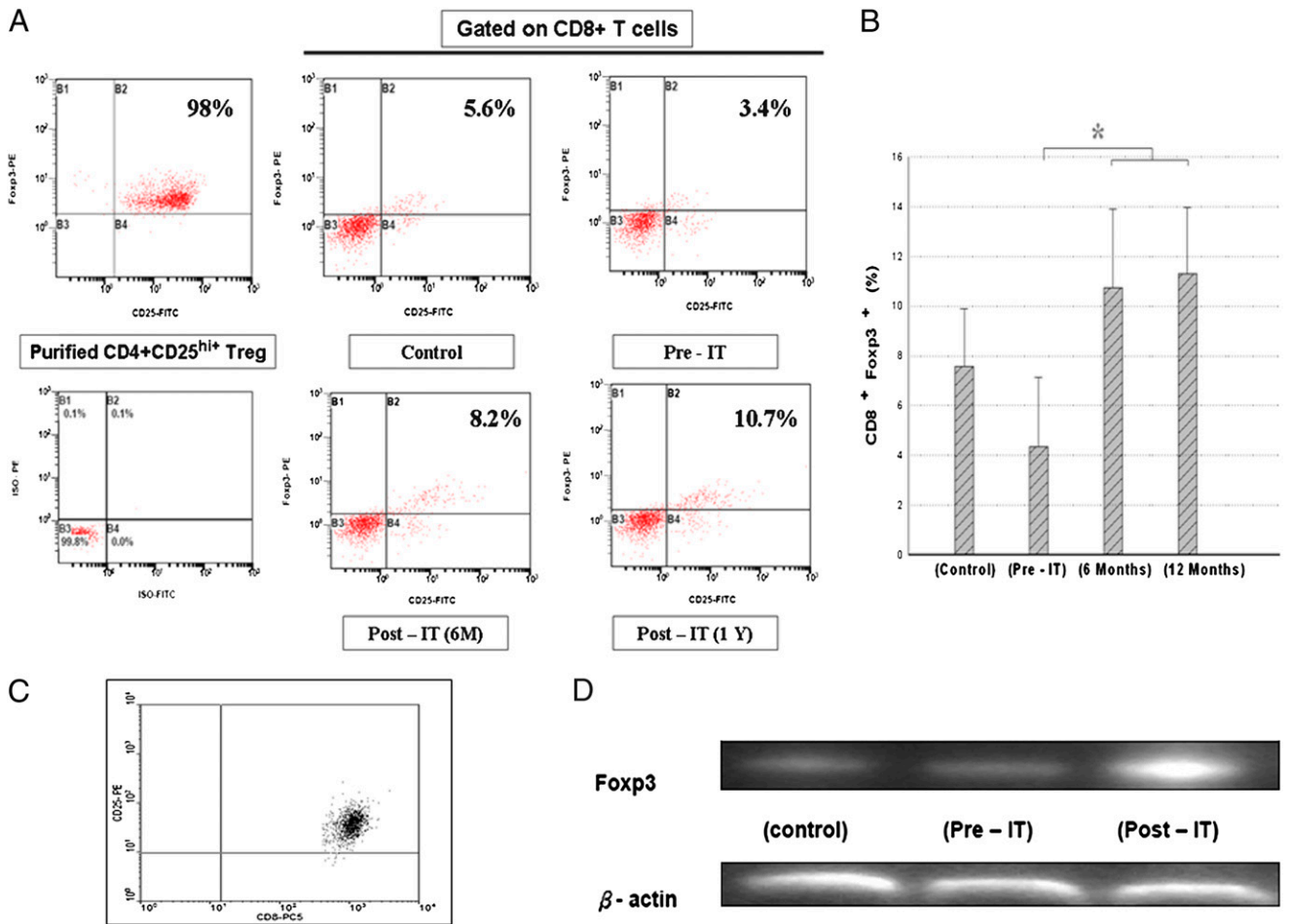
Pam3CSK4 enhanced *D. pteronyssinus* 2-induced increase in CD8<sup>+</sup> Treg cells

Freshly isolated PBMCs were cultured with *D. pteronyssinus* 2 in the presence or absence of Pam3CSK4 for 5 d. The number of

CD8<sup>+</sup>Foxp3<sup>+</sup> T cells was greater in nonatopic subjects than in asthmatic subjects before IT without Pam3CSK4 stimulation (6.36% ± 1.20% versus 3.73% ± 1.05%, respectively; *p* < 0.05) (Fig. 3B). After Pam3CSK4 costimulation with *D. pteronyssinus* 2, the numbers of CD8<sup>+</sup>Foxp3<sup>+</sup> T cells in the asthmatic group were much greater than with *D. pteronyssinus* 2 alone (7.93% ± 1.09% versus 3.42% ± 1.70%, respectively; *p* < 0.05). Pam3CSK4 costimulation also increased the percentage of CD8<sup>+</sup>Foxp3<sup>+</sup> cells in nonatopic children (10.24% ± 1.8% versus 4.65% ± 1.29%; *p* < 0.05) (Fig. 3B).

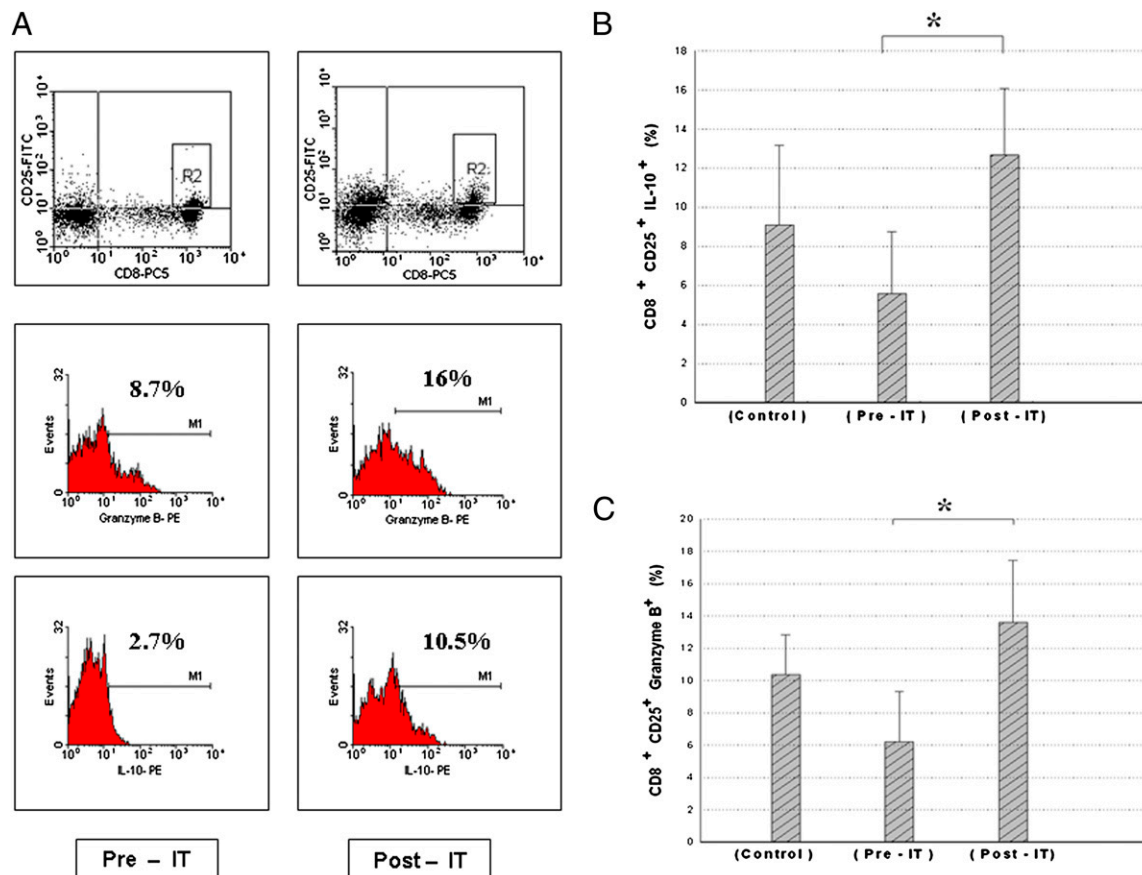
Pam3CSK4 increases expression of granzyme B and IL-10 in CD8<sup>+</sup> Treg cells

In a summary from 30 paired experiments in asthmatic subjects, costimulation with Pam3CSK4 and *D. pteronyssinus* 2 led to



**FIGURE 1.** Fcpx3 expression in CD8<sup>+</sup> cells during *D. pteronyssinus*-specific IT. **A**, PBMCs from asthmatic children during IT were stimulated with *D. pteronyssinus* 2 for 5 d, and cells with intracellular expression of Fcpx3 were analyzed for CD8<sup>+</sup>CD25<sup>+</sup> T cells. Representative figures are shown. **B**, Intracellular Fcpx3 expression was assessed in CD8<sup>+</sup> Treg cells from normal (*n* = 30) and asthmatic subjects (*n* = 30) before IT, as well as 6 and 12 mo after treatment. \**p* < 0.05. **C**, Purified CD8<sup>+</sup>CD25<sup>+</sup> T cells after *D. pteronyssinus* 2-stimulated PBMCs were analyzed by flow cytometry. **D**, Increased expression of Fcpx3 by purified CD8<sup>+</sup>CD25<sup>+</sup> T cells was observed during IT by Western blot analysis. Ten independent experiments were performed, with essentially identical results.





**FIGURE 2.** Intracellular IL-10 and granzyme B levels in CD8<sup>+</sup>CD25<sup>+</sup> Treg cells before and after IT. PBMCs were stimulated with *D. pteronyssinus* 2 for 5 d, followed by stimulation with PMA (10 ng/ml) plus ionomycin (1  $\mu$ g/ml) for the last 5 h and the addition of brefeldin A (10  $\mu$ g/ml) for the final hour. A, Intracellular expression of IL-10 and granzyme B was measured by R2 gating in CD8<sup>+</sup>CD25<sup>+</sup> T cells using flow cytometry. The results of 30 paired experiments for IL-10 (B) and granzyme B (C) production by PBMCs is shown. \* $p < 0.05$ .

a significant increase in CD8<sup>+</sup>CD25<sup>+</sup>IL-10<sup>+</sup> T cells compared to stimulation with *D. pteronyssinus* 2 alone ( $19.96\% \pm 3.66\%$  versus  $5.44\% \pm 2.9\%$ , respectively;  $p < 0.05$ ) (Fig. 3C). Costimulation with Pam3CSK4 and *D. pteronyssinus* 2 also significantly increased granzyme B expression in CD8<sup>+</sup>CD25<sup>+</sup> T cells ( $19.29\% \pm 5.14\%$  versus  $9.33\% \pm 3.61\%$ ;  $p < 0.05$ ) in nonatopic children (Fig. 3D).

#### *Pam3CSK4 modulates D. pteronyssinus 2-induced Th2 cytokine profiles*

PBMCs from asthma patients before IT produced more IL-4 than did those from nonatopic children (Fig. 4A). Pam3CSK4 significantly suppressed *D. pteronyssinus* 2-induced IL-4 production by PBMCs from asthma patients and nonatopic controls (Fig. 4A). The production of IFN- $\gamma$ , a Th1 cytokine, was measured to elucidate whether the ability of Pam3CSK4 to inhibit Th2 cytokine production may be associated with deviation toward a Th1 immune response. In nonatopic subjects, but not in asthmatic subjects, IFN- $\gamma$  production was markedly greater in Pam3CSK4-treated PBMCs compared with *D. pteronyssinus* 2-treated cells ( $p < 0.05$ ) (Fig. 4B). Pam3CSK4 stimulation elicited greater IL-10 cytokine production by PBMCs from asthma patients and nonatopic controls (Fig. 4C).

#### *Suppressive activity of CD8<sup>+</sup> Treg cells involves cell contact*

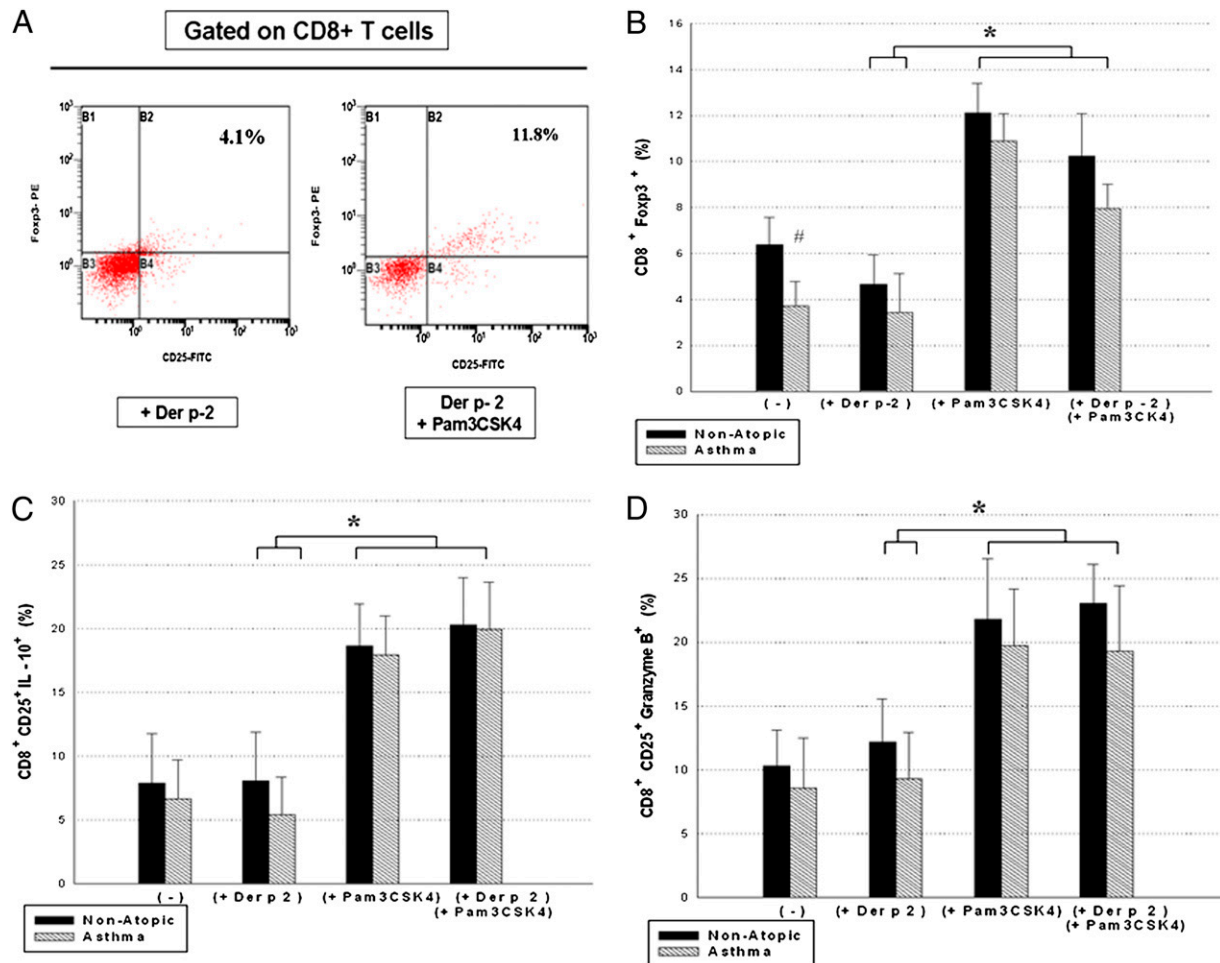
Next, experiments were performed to determine whether CD8<sup>+</sup>CD25<sup>+</sup> Treg cells regulate CD4<sup>+</sup> T cell proliferation induced by Pam3CSK4 stimulation. PBMCs and CD8<sup>+</sup>-depleted PBMCs from asthma patients before IT and nonatopic controls were stim-

ulated with Pam3CSK4 and then labeled with CFSE. Purified, non-CFSE-labeled CD8<sup>+</sup>CD25<sup>+</sup> Treg cells were added to CD8<sup>+</sup>-depleted PBMCs, and cell proliferation was measured. In a representative study, CD8<sup>+</sup>CD25<sup>+</sup> Treg cells suppressed the proliferation of CD4<sup>+</sup> T cells after stimulation with Pam3CSK4 (Fig. 5A). Fig. 5B summarizes the data from 20 asthmatic patients; CD4<sup>+</sup> T cell proliferation induced by anti-CD3 was enhanced following CD8<sup>+</sup> T cell depletion, and the addition of CD8<sup>+</sup>CD25<sup>+</sup> Treg cells significantly inhibited proliferation. The suppression of CD4<sup>+</sup> T cell proliferation by CD8<sup>+</sup>CD25<sup>+</sup> Treg cells was also observed in cells stimulated with Pam3CSK4 (Fig. 5B).

Transwell assay was used to determine whether the Pam3CSK4-mediated suppression of T cell proliferation required cell-cell contact or occurred via soluble factors. Incubation of CD8<sup>+</sup> Treg cells and CD8<sup>+</sup>-depleted PBMCs costimulated with Pam3CSK4 in separate chambers of the Transwell revealed no inhibition of T cell proliferation; the results were similar to those observed when CD4<sup>+</sup> T cells were cocultured with CD8<sup>+</sup>-depleted PBMCs (Fig. 5C). In summary, CD8<sup>+</sup> Treg cell-mediated suppression was largely dependent on cell contact.

#### *CD8<sup>+</sup>CD25<sup>+</sup> Treg cells involved in apoptosis of CD4<sup>+</sup>CD45RO<sup>hi</sup> cells*

Our study probed CD8<sup>+</sup>CD25<sup>+</sup> Treg cells triggering CD4<sup>+</sup>CD45RO<sup>hi</sup> cell apoptosis by *D. pteronyssinus* 2 during IT. The effect of CD4<sup>+</sup> and CD8<sup>+</sup> Treg cells on CD4<sup>+</sup>CD45RO<sup>hi</sup> apoptosis was assessed by determining the percentage of TUNEL<sup>+</sup>CD4<sup>+</sup>CD45RO<sup>hi</sup> T cells in CD25<sup>+</sup>-depleted PBMCs co-



**FIGURE 3.** Foxp3 expression and intracellular IL-10 and granzyme B levels in CD8<sup>+</sup>CD25<sup>+</sup> Treg cells stimulated with Pam3CSK4. PBMCs were stimulated with Pam3CSK4 and/or *D. pteronyssinus* 2 for 5 d. Summary of 30 paired experiments for intracellular Foxp3 expression in CD8<sup>+</sup> Treg cells (A) and CD4<sup>+</sup> Treg cells (B) from asthmatic subjects before IT and nonatopic subjects. Intracellular expression of IL-10 (C) and granzyme B (D) were measured in CD8<sup>+</sup>CD25<sup>+</sup> T cells using flow cytometry. #*p* < 0.05; asthmatic versus nonatopic subjects; \**p* < 0.05; between each treatment group.

cultured with purified CD8<sup>+</sup>CD25<sup>+</sup> or CD4<sup>+</sup>CD25<sup>+</sup> Treg cells. The percentage of TUNEL<sup>+</sup>CD4<sup>+</sup>CD45RO<sup>hi</sup> T cells increased after the addition of CD8<sup>+</sup>CD25<sup>+</sup> Treg cells (but not after the addition of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells) to CD25<sup>+</sup>-depleted PBMCs from control subjects and asthmatic patients (Table III). We also confirmed, by Annexin V-PI labeling, that the apoptosis of CD45RO cells was greater after adding CD8 Treg cells than after adding CD4 Treg cells during IT, as shown in Fig. 6.

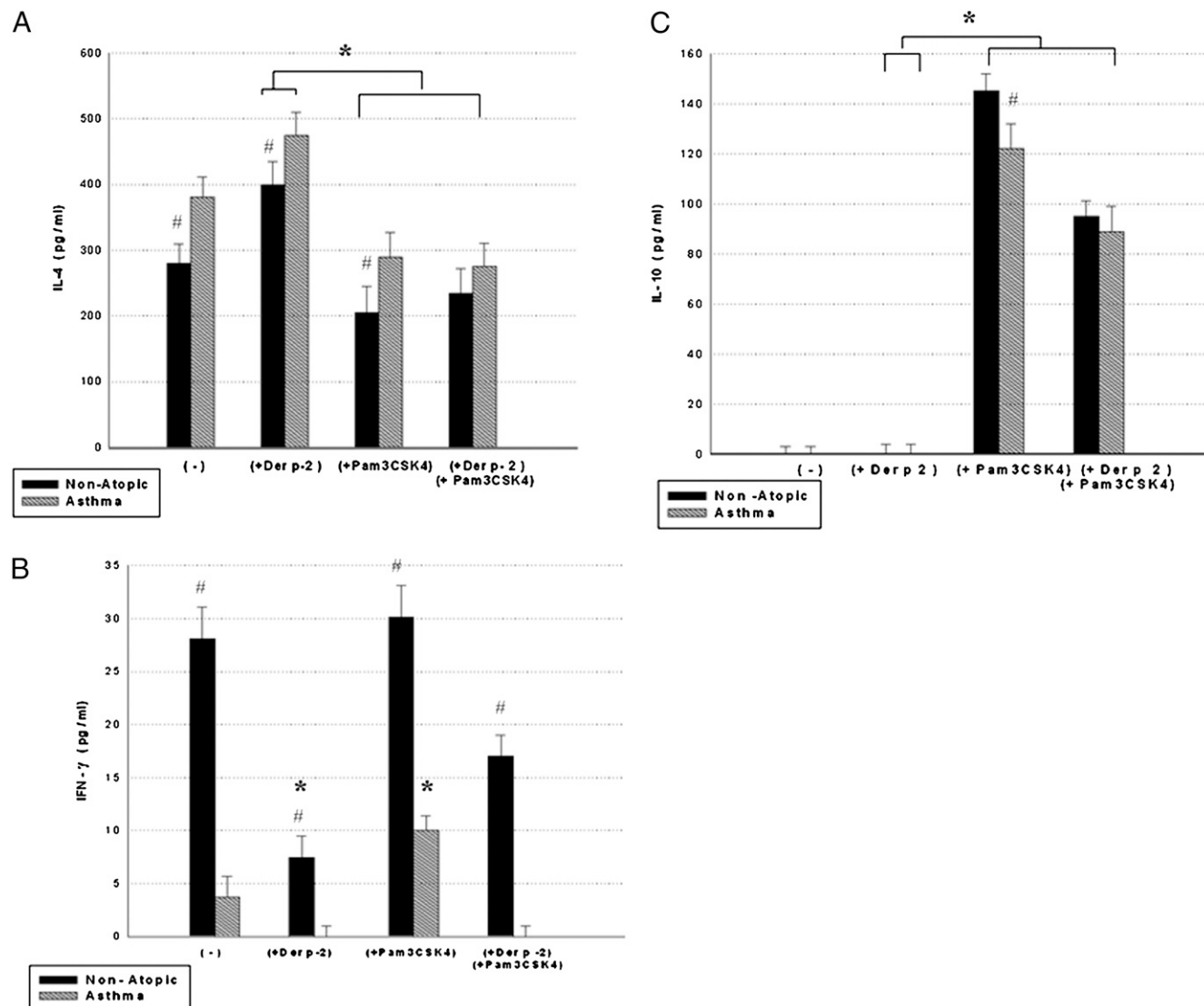
## Discussion

This study demonstrated that *D. pteronyssinus*-specific IT induced CD8<sup>+</sup>Foxp3<sup>+</sup> Treg cells with increase of CD8<sup>+</sup>CD25<sup>+</sup>IL-10<sup>+</sup> and CD8<sup>+</sup>CD25<sup>+</sup>granzyme B<sup>+</sup> cells may partially account for CD8<sup>+</sup> Treg cell suppressive activity. The data further support the crucial role of the synthetic TLR2 agonist Pam3CSK4 in preventing a Th2 cell-mediated allergic immune response by increasing CD8<sup>+</sup>CD25<sup>+</sup> Treg cells to suppress T cell proliferation and increase CD4<sup>+</sup>CD45RO<sup>+</sup> cell apoptosis. CD8<sup>+</sup> Treg cells inhibit T cell proliferation by cell-cell contact and increase the production of suppressive cytokine IL-10. Taken together, the results suggest that Pam3CSK4 stimulation plays a key role in limiting Th2 cell-mediated allergic immune response by decreasing IL-4 production and increasing Treg cell function.

With relatively small numbers of CD8<sup>+</sup> Treg cells in peripheral blood, CD8<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells can be generated by continuous

Ag stimulation (12, 13). CD8<sup>+</sup> Treg cells were first identified in human tonsils; upon in vitro activation, Foxp3<sup>+</sup>CD8<sup>+</sup> Treg cells were shown to inhibit T cell proliferation directly (12). CD8<sup>+</sup> Treg cells seem to perform a regulatory function that limits autoimmune disease in several experimental models (17, 31, 32). Systemic immunization with allergen in mice induces CD8<sup>+</sup> Treg cells that can inhibit the development of allergic diarrhea, suggesting that CD8<sup>+</sup> Treg cells may play a pivotal role in limiting allergic disease (33). In this study, we demonstrated that functional CD8<sup>+</sup> Treg cells in vitro stimulation by *D. pteronyssinus* 2 for 5 d has not shown the effect on CD8 Treg cells, possibly as the result of a shorter treatment period of culture. *D. pteronyssinus* IT, by repeated Ag stimulation, may augment the CD8<sup>+</sup> Treg population and amplify the mechanism of immune tolerance.

TLR2 provides an important link between innate and adaptive immunity, particularly by modulating the Th2 response in atopic individuals (34, 35). However, there are conflicting results regarding which mechanisms are involved in the modulation of the Th1/Th2 balance in experimental allergic airway disease, depending on the timing of antigenic stimulation, the dosage of different TLR2 agonists, and the genetic background of animal models. Pam3CSK4 engagement directly triggers Th1 cells (inducing IFN- $\gamma$  production and CD8<sup>+</sup> T cell proliferation) but not Th2 cells (4). Pam3CSK4 reverses established OVA-induced airway inflammation by a mechanism that is critically dependent on IL-12 but not IL-10 or TGF- $\beta$



**FIGURE 4.** Production of IL-4 and -10 and IFN- $\gamma$  by PBMCs costimulated with Pam3CSK4 and/or *D. pteronyssinus* 2. Supernatants from PBMCs stimulated with Pam3CSK4 and/or *D. pteronyssinus* 2 for 5 d were collected for analyses of IL-4 (A), IFN- $\gamma$  (B), and IL-10 (C) production. # $p$  < 0.05; asthmatic versus nonatopic subjects; \* $p$  < 0.05; between each treatment group.

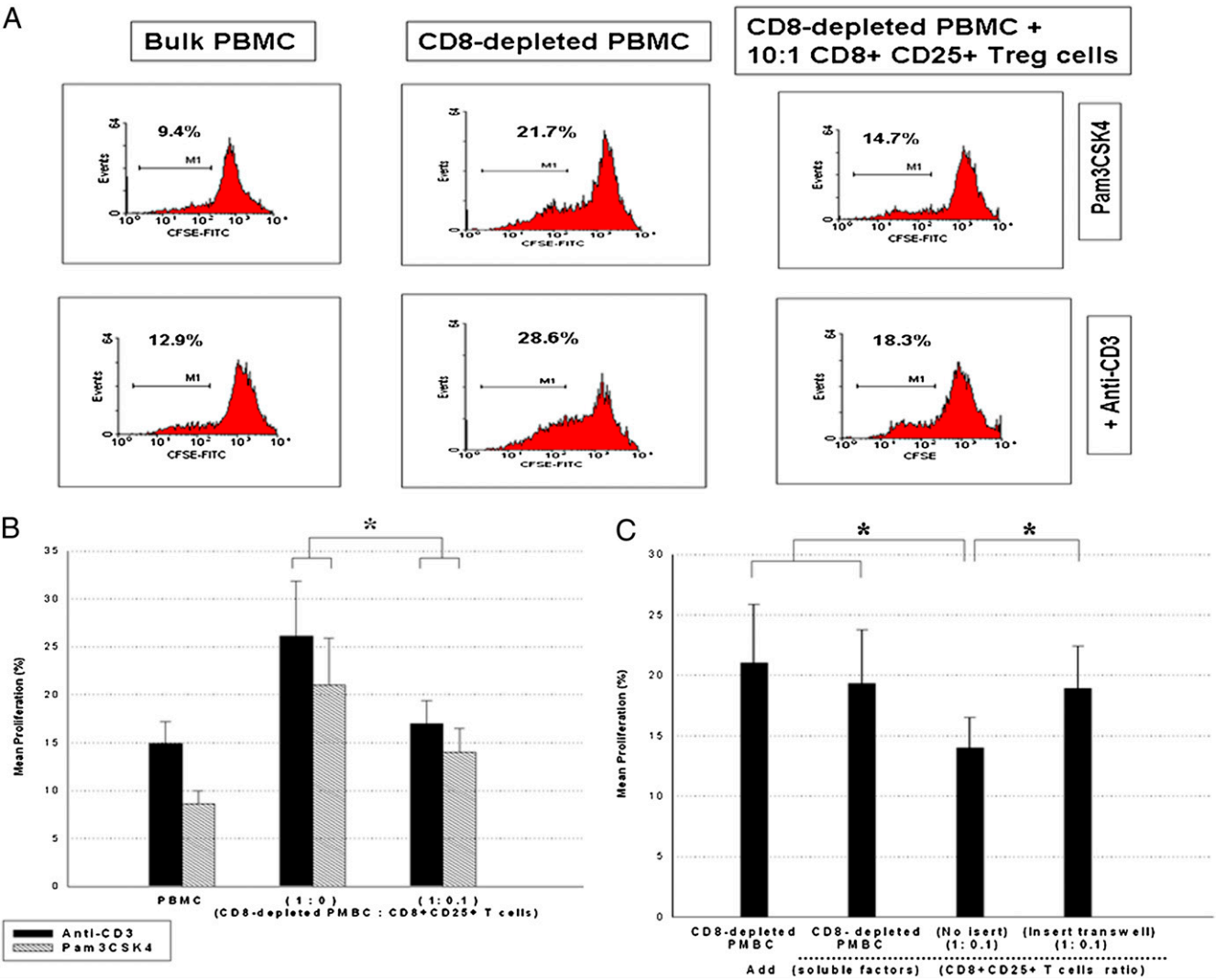
(36). Pam3CSK4 suppresses eosinophil infiltration in murine allergic conjunctivitis by inducing CD4<sup>+</sup> T cell apoptosis rather than by upregulating Th1 responses (37). Another anti-inflammatory mechanism indicates that Pam3CSK4 directly activates CD4<sup>+</sup>CD25<sup>+</sup> Treg cell expansion and suppressive function (5, 6). In a murine model of asthma, Pam3CSK4 was shown to be a valid candidate adjuvant for sublingual allergy vaccines that mediated Th1/Treg cell responses (30). In the present study, we first demonstrated in humans that Pam3CSK4 activates CD8<sup>+</sup>Foxp3<sup>+</sup> Treg cells to suppress CD4<sup>+</sup> proliferation, as well as decrease IL-4 production and increase IL-10 production.

Apoptosis of allergen-specific Th2 cells during IT represents a unique downregulatory mechanism that prevents the continuous activation of Th2 immune responses by allergen (26, 27). We showed that CD8<sup>+</sup> Treg cells, but not CD4<sup>+</sup> Treg cells, could enhance CD4<sup>+</sup>CD45RO<sup>hi</sup> cell apoptosis. Cell contact with CD8<sup>+</sup> Treg cells expressing increased granzyme B may induce cell apoptosis of CD4<sup>+</sup>CD45RO<sup>+</sup> memory T cells during IT. In support of our findings, a functional study in autoimmune hepatitis subjects (38) revealed that CD4<sup>+</sup>CD25<sup>hi</sup> Treg cells act through direct

contact with target cells by modifying levels of regulatory cytokines but not by inducing target cell apoptosis.

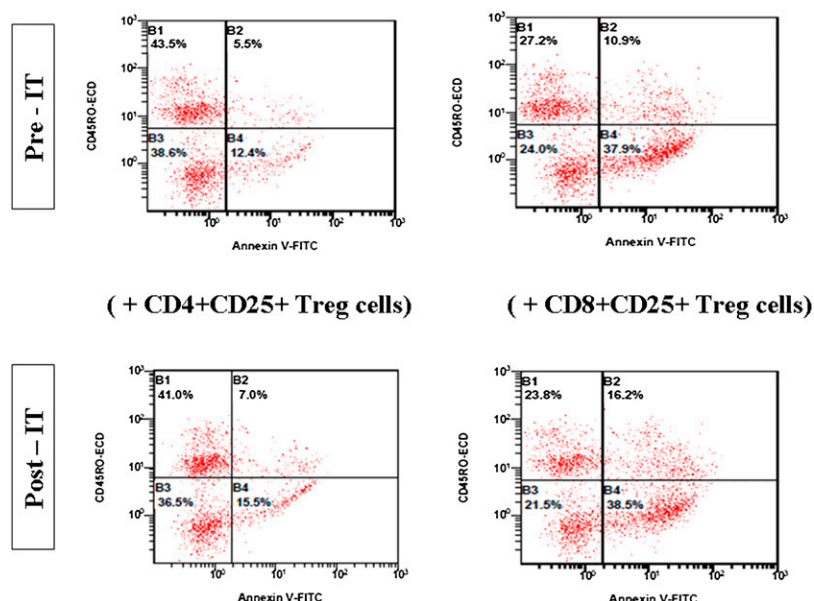
Some studies also showed that CD4<sup>+</sup>CD25<sup>+</sup> Treg cells are less able to suppress Th2 responses compared with Th1 responses (39–42). Grindebacke et al. (39) first demonstrated that allergen-stimulated CD4<sup>+</sup> Treg cells during IT failed to suppress Th2 responses, despite increased IL-10 production by T cells. Human thymus-derived CD4<sup>+</sup>CD25<sup>+</sup> T cell clones suppress Th1 clone proliferation better than Th2 clone proliferation (40). The therapeutic transfer of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells only partially suppressed Th2-induced disease in an autoimmune gastritis model (41). Human purified CD4<sup>+</sup>CD25<sup>hi</sup> Treg cells isolated from PBMCs of control and cancer patients suppressed proliferation but did not mediate apoptosis in autologous CD4<sup>+</sup>CD25<sup>+</sup> responder cells (42).

However, the interaction between the two subsets of Treg cells that protect against allergy remains unclear. Adoptive transfer of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells into sensitized mice resulted in the suppression of lung allergic responses. In CD8 knockout recipient mice, transferred Treg cells restored airway inflammation following allergen exposure (43). In addition, it was shown that Foxp3-expressing





**FIGURE 6.** Double-fluorescence study by flow cytometry of CD45RO lymphocyte subpopulations and apoptosis during IT. CD25<sup>+</sup>-depleted PBMCs were cocultured with CD4<sup>+</sup>CD25<sup>+</sup> Treg cells or CD8<sup>+</sup>CD25<sup>+</sup> Treg cells with *D. pteronyssinus* 2 stimulation for 5 d. Apoptosis in these cells was simultaneously determined by Annexin V labeling and negative PI gating. Representative graphs during IT with similar results ( $n = 10$ ).



support the idea that Pam3CSK4 may act as a candidate adjuvant for therapeutic intervention in allergic diseases. Future studies to understand how Pam3CSK4 affects TLR2 signaling may offer more specific targets to modulate the balance among Th1, Th2, and Treg cells in allergy and other immune diseases.

## Disclosures

The authors have no financial conflicts of interest.

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