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Annotating PDFs

Version 1.2 with The Author Center October 21, 2009

1. Introduction

eProof files are self-contained PDF documents for viewing on-screen and for printing. They contain all appropriate formatting and fonts to ensure correct rendering on-screen and when printing hardcopy. DJS sends eProofs that can be viewed, annotated, and printed using the free version of Adobe Reader 7 (or greater). These eProofs are "enabled" with commenting rights, therefore they can be modified by using special markup tools in Adobe Reader that are not normally available unless using the Standard or Professional version of Adobe Acrobat.

The screen images in this document were captured on a PC running Adobe Reader version 8.1.0. Though some of the images may differ in appearance from your platform/version, the basic functionality remains similar. At the time of this writing, Acrobat Reader v8.1.0 is freely available and can be downloaded from: http://www.adobe.com/products/acrobat/readstep2.html

2. The Author Center process

A. You will receive an email that contains a link to The Author Center:
For example: http://authorcenter.dartmouthjournals.com/<random key link to proof>

B. Click on the link to visit The Author Center.

C. Follow the directions provided by The Author Center to access the proof.

D. Save a copy of the proof to your desktop or to a folder on your hard drive.

E. Make corrections using Acrobat's Comment & Markup tools.

F. Save the PDF file, now with annotations, and use the same link provided to return your proof via The Author Center.

3. Comment & Markup toolbar functionality

A. Sticky Note tool; B. Text Edits tool; C. Stamp tool; D. Highlight Text tool; E. Callout tool; F. Text Box tool; G. Various Object tools; H. Pencil tool

A. Show the Comment & Markup toolbar

The Comment & Markup toolbar doesn't appear by default. Do one of the following:

- Select View > Toolbars > Comment & Markup.
- Select Tools > Comment & Markup > Show Comment & Markup Toolbar.
- Click the Review & Comment button in the Task toolbar, and choose Show Comment & Markup Toolbar.

To add or remove tools for this toolbar, right-click the toolbar and select the tool. Or, select Tools > Customize Toolbars.

B. Select a commenting or markup tool

Do one of the following:

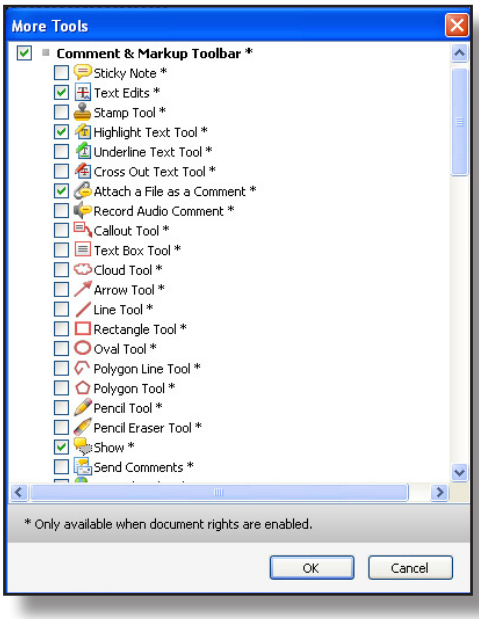
- Select a tool from the Comment & Markup toolbar.
- Select Tools > Comment & Markup > [tool].

Note: After an initial comment is made, the tool changes back to the Select tool so that the comment can be moved, resized, or edited. (The Pencil, Highlight Text, and Line tools stay selected.)

C. Keep a commenting tool selected

Multiple comments can be added without reselecting the tool. Select the tool to use (but don't use it yet).

- Select View > Toolbars > Properties Bar.
- Select Keep Tool Selected.



Choose Tools > Customize Toolbars to remove unnecessary items from the toolbar (see Section 8 for suggested toolbar layout)

4. The Properties bar

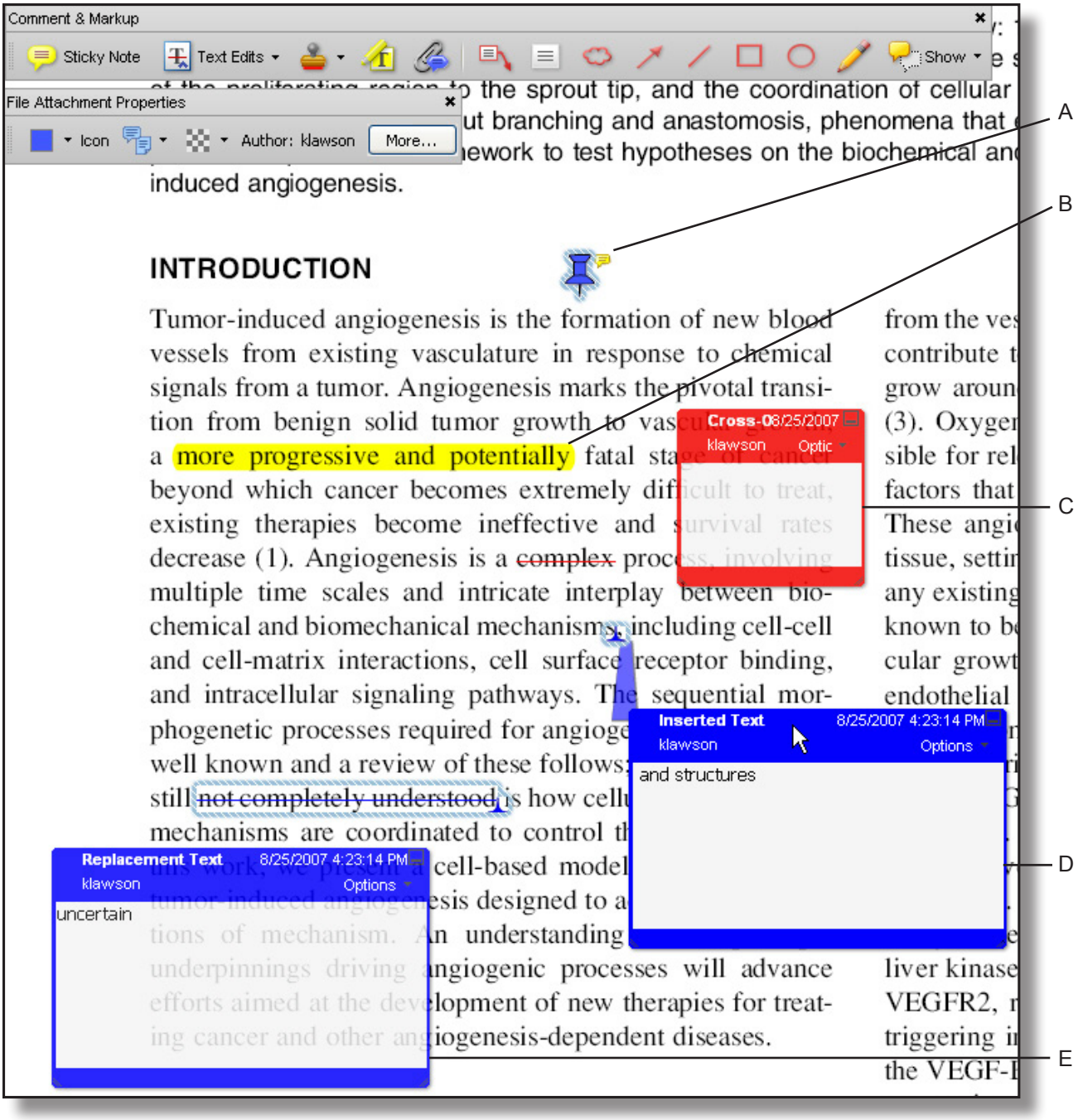
The Properties bar can be used to format text and select options for individual tools.

To view the Properties bar, do one of the following:

- Choose View > Toolbars > Properties Bar.
- Right-click the toolbar area; choose Properties Bar.
- Select [Ctrl-E]

5. Using the comment and markup tools

To *insert*, *delete*, or *replace* text, use the **Text Edits** tool. Select the Text Edits tool, then select the text with the cursor (or simply position it) and begin typing. A pop-up note will appear based upon the modification (e.g., inserted text, replacement text, etc.). Use the Properties bar to format text in pop-up notes. A pop-up note can be minimized by selecting the ⊟ button inside it.



A. Attached file; B. Highlighted text; C. Crossed-out (strike-through) text; D. Inserted text; E. Replaced text

6. Inserting symbols or special characters

An 'insert symbol' feature is not available for annotations, and copying/pasting symbols or non-keyboard characters from Microsoft Word does not always work. Use angle brackets < > to indicate these special characters (e.g., <alpha>, <beta>).

7. Editing near watermarks and hyperlinked text

eProof documents often contain watermarks and/or hyperlinked text. Selecting characters near these items can be difficult using the mouse alone. To edit an eProof which contains text in these areas, do the following:

- Without selecting the watermark or hyperlink, place the cursor near the area for editing.
- Use the arrow keys to move the cursor beside the text to be edited.
- Hold down the shift key while simultaneously using arrow keys to select the block of text, if necessary.
- Insert, replace, or delete text, as needed.

8. Summary of main functions

Insert text - Use Text Edits tool (position cursor and begin typing)
Replace text - Use Text Edits tool (select text and begin typing)
Delete text - Use Text Edits tool (select text and press delete key)
Highlight text - Use Highlight Text tool (select text)
Attach a file - Use the Attach a File with Comment tool (select tool, position cursor and click mouse, select file)

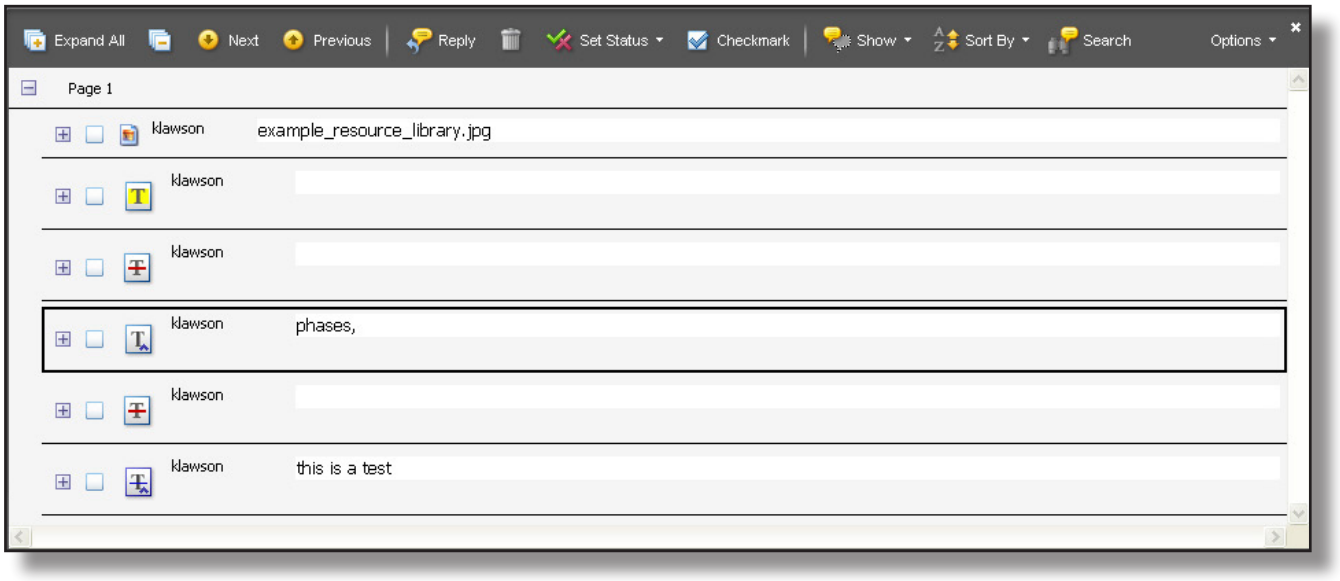
Suggested toolbar layout

9. Reviewing changes

To review all changes, do the following:

- Select the Show button on the Comment & Markup toolbar.
- Select Show Comments List.

Note: *Selecting a correction in the list will highlight the corresponding item in the document, and vice versa.*



Use the Comments list to review all changes

TLR2 Agonists Enhance CD8$^+$Foxp3$^+$ 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§

Pam3CSK4, a synthetic TLR2 ligand, has been shown to expand CD4$^+$ regulatory T cells (Treg cells). Less is known about the function of CD8$^+$ Treg cells than about the function of CD4$^+$ Treg cells generated during allergen-specific immunotherapy (IT). This study investigated whether *Dermatophagoides pteronyssinus*-specific IT could expand the CD8$^+$CD25$^+$Foxp3$^+$ 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$^+$Foxp3$^+$ Treg cells expressing intracellular IL-10 and granzyme B. Costimulation of PBMCs with Pam3CSK4 and *D. pteronyssinus* 2 expanded the CD8$^+$CD25$^+$Foxp3$^+$ Treg population and inhibited *D. pteronyssinus* 2-induced IL-4 production. Pam3CSK4-treated CD8$^+$CD25$^+$ Treg cells directly suppressed CD4$^+$ T cell proliferation by cell-contact inhibition. TUNEL revealed that CD8$^+$CD25$^+$ Treg cells, but not CD4$^+$CD25$^+$ Treg cells, directly induced CD4$^+$CD45RO^{hi+} apoptosis. Our results provide direct evidence that Pam3CSK4 induces an immunomodulatory effect by inducing CD8$^+$ Treg cells; therefore, it may be a good adjuvant for the treatment of mite allergies. *The Journal of Immunology*, 2010, 184: 000–000.

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$^+$CD25$^+$ 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$^+$ Treg cells' involvement in maintaining self-tolerance was recently identified (8, 9). These cells' surface markers include CD25, CD103, and CD122 (10, 11). CD8$^+$ Treg cells with regulatory function express transcription factor Foxp3 (11–13). Human CD8$^+$ 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 microenvironment, CD8$^+$CD25$^+$ Treg cells have a suppressive ability that typically is associated with CD4$^+$ Treg cells (19–21).

Unlike CD4$^+$Foxp3$^+$ Treg cells generated in the thymus, the suppressive CD8$^+$Foxp3$^+$ Treg cells appear after primary Ag stimulation, suggesting that they are amplified by TCR stimulation (22). CD8$^+$ Treg cells can suppress cellular proliferation of CD4$^+$ naive and effector T cells via cell–cell contact lysis or soluble factors, such as IL-10 and TGF-β (23). Cottalorda et al. (24) demonstrated that TLR2 engagement on CD8$^+$ cells induced a sustained expression of CD25, with an increase in Treg function. However, it is not clear whether CD8$^+$ 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$^+$ 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$^+$ Treg populations, as well as whether Pam3CSK4 increases CD8$^+$Foxp3$^+$ 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 (≥2+) and an IgE-specific test greater than third grade (>3.5 kU/l) using the CAP system (Pharmacia Biotech, 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

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Abbreviations used in this paper: IT, immunotherapy; PI, propidium iodide; Treg cell, regulatory T cell.

followed for 1 y (27). Patients were instructed to record asthmatic scores (27), and pulmonary function (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, ECD, 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 1×10^6 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 (BioWhittaker, Walkersville, MD). In some experiments, $CD8^+$ or $CD4^+$ cells were depleted directly from PBMCs using microbeads, according to manufacturer's protocol (BD Biosciences). $CD8^+CD25^+$ T cells were isolated using a $CD8^+$ T cells enrichment kit, followed by separation with CD25 microbeads (BD Biosciences). The purity of the $CD8^+CD25^+$ T cell population analyzed by flow cytometry was >95%. $CD4^+CD25^{hi}$ 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 $CD8^+CD25^+$ Treg cells (5×10^5) for Western blotting. Purified $CD8^+CD25^+$ 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 ($CD8^+CD25^+$ 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. $CD8^+CD25^+$ 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 $CD8^+$-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), $CD8^+CD25^+$ Treg cells were added to the culture autologous, CD8-depleted, and CFSE-labeled PBMCs at a 1:10 ratio, and $CD4^+$ 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 $CD8^+$-depleted PBMCs and $CD8^+CD25^+$ Treg cells were placed in the upper chamber, and the CFSE-labeled $CD8^+$-depleted PBMCs were placed in the lower chamber. The culture supernatant of $CD8^+CD25^+$ Treg cells and PBMCs was added to CFSE-labeled $CD8^+$-depleted PBMCs culture to confirm the inhibitory effect of soluble factors. After culturing, the proliferation of $CD4^+$ T cells was assessed by CFSE fluorescence with flow cytometric analysis.

Detection of $CD4^+CD45RO^+$ T cell apoptosis

To assess whether the presence of $CD4^+$ or $CD8^+$ Treg cells affects the apoptosis of $CD4^+CD45RO^+$ T cells during IT, purified $CD4^+CD25^+$ or $CD8^+CD25^+$ Treg cells were added to autologous $CD25^+$-depleted PBMCs with *D. pteronyssinus* 2 stimulation for 5 d. The apoptosis rate of $CD4^+CD45RO^+$ 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 ± SD. Differences between the means before and after IT were analyzed used the paired Student *t* test. Differences of means compared with each group were analyzed using ANOVA, followed by the Duncan test. A p value < 0.05 was considered significant.

Results

Increase in $CD8^+$ 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). T1, T2

Foxp3 is the essential transcription factor for the suppressor function of Treg cells. To study whether $CD8^+$ Treg cells were induced by specific *D. pteronyssinus* IT, Treg cells were analyzed by flow cytometry for surface markers and intracellular Foxp3 expression simultaneously. $CD8^+CD25^+Foxp3^+$ cells increased after IT of asthmatic subjects (Fig. 1*A*). F1 The number of $CD8^+Foxp3^+$ cells in *D. pteronyssinus* 2-stimulated PBMCs had increased after 6 mo and 1 year of IT (4.35% ± 2.38% versus 10.75% ± 3.14% and 11.30% ± 2.65%; before IT versus after 6 and 12 mo of IT, respectively; Fig. 1*B*). Purified $CD8^+CD25^+$ T cells after *D. pteronyssinus* 2-stimulated PBMCs were analyzed by flow cytometry, and representative results are shown (Fig. 1*C*). Increased expression of Foxp3 by purified $CD8^+CD25^+$ T cells by Western blot analysis was observed during IT (Fig. 1*D*), confirming the flow cytometry results.

D. pteronyssinus-*specific IT increased $CD8^+CD25^+$ Treg cells expressing granzyme B and IL-10*

Experiments were performed to investigate whether the increase in $CD8^+CD25^+$ Treg cells was associated with IL-10 and granzyme B expression. *D. pteronyssinus* 2-stimulated PBMCs were 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 $CD8^+CD25^+$ T cells (Fig. 2*A*). F2

Table I. *Patient characteristics*

	IT Group	Control Group
Patients (*n*)	50	50
Age (y; mean ± SD)	12.15 ± 3.56	12.06 ± 3.71
Gender (male:female)	26:24	30:20
Skin prick test (grade)	3.7 ± 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)
D. pteronyssinus-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.

PBMCs from asthmatic patients contained greater numbers of $CD8^+CD25^+IL\text{-}10^+$ T cells after 1 y of IT than before IT (12.69% ± 3.39% versus 5.58% ± 3.16%, respectively; $p < 0.05$) (Fig. 2*B*) as well as greater numbers of granzyme B-expressing $CD8^+CD25^+$ T cells (13.59% ± 3.85% versus 6.22% ± 3.10%, respectively; $p < 0.05$) (Fig. 2*C*). This suggests that $CD8^+$ Treg cells contribute to successful treatment.

Pam3CSK4 enhanced D. pteronyssinus *2-induced increase in $CD8^+$ Treg cells*

Freshly isolated PBMCs were cultured with *D. pteronyssinus* 2 in the presence or absence of Pam3CSK4 for 5 d. The number of $CD8^+Foxp3^+$ 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. 3*B*). After Pam3CSK4 costimulation with *D. pteronyssinus* 2, the numbers of $CD8^+Foxp3^+$ 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 $CD8^+Foxp3^+$ cells in nonatopic children (10.24% ± 1.8% versus 4.65% ± 1.29%; $p < 0.05$) (Fig. 3*B*).

Pam3CSK4 increases expression of granzyme B and IL-10 in $CD8^+$ Treg cells

In a summary from 30 paired experiments in asthmatic subjects, costimulation with Pam3CSK4 and *D. pteronyssinus* 2 led to a significant increase in $CD8^+CD25^+IL\text{-}10^+$ T cells compared to stimulation with *D. pteronyssinus* 2 alone (19.96% ± 3.66% versus 5.44% ± 2.9%, respectively; $p < 0.05$) (Fig. 3*C*).

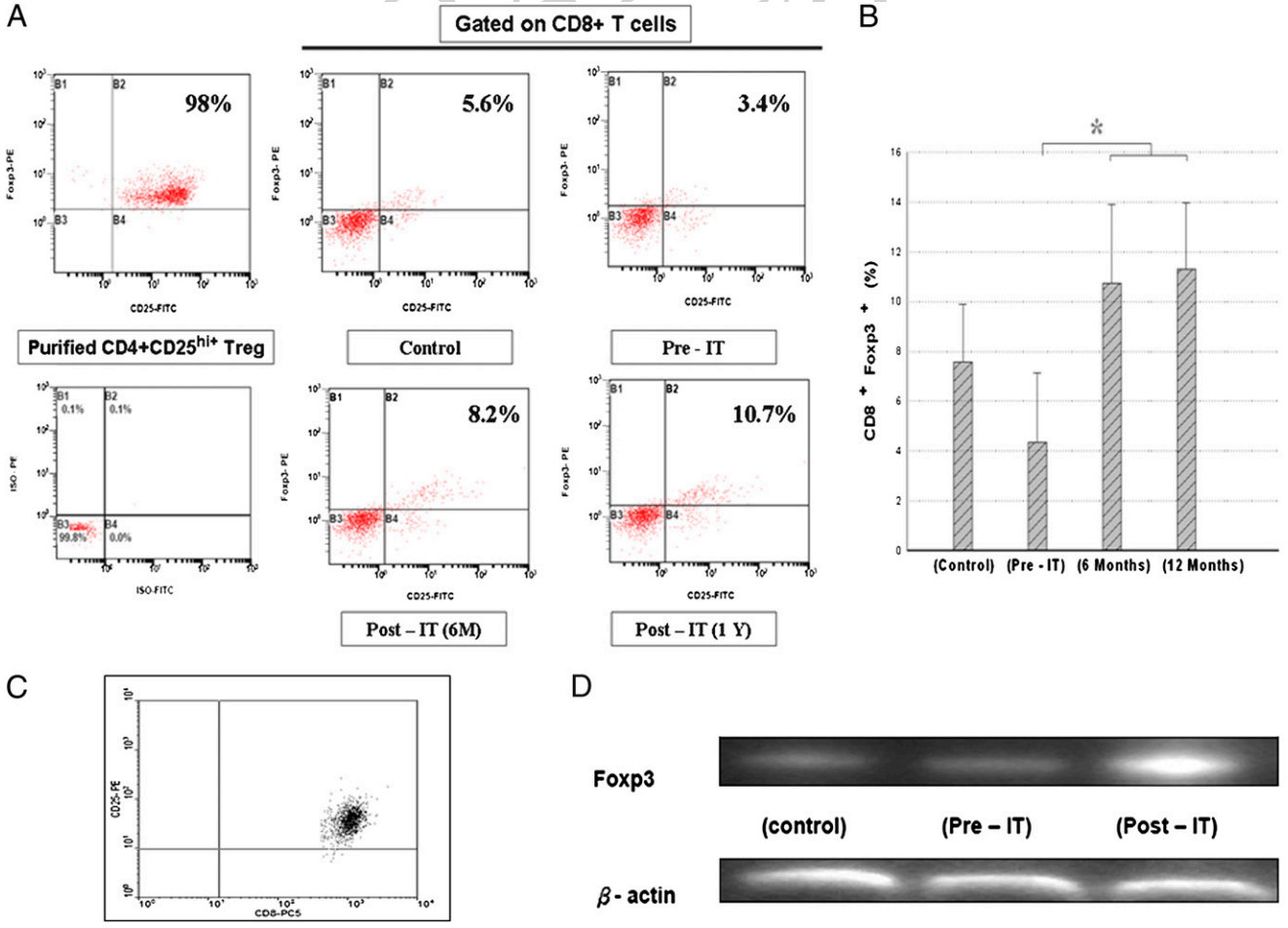

FIGURE 1. Foxp3 expression in $CD8^+$ cells during *D. pteronyssinus*-specific IT. *A*, PBMCs from asthmatic children during IT were stimulated with *D. pteronyssinus* 2 for 5 d, and cells with intracellular expression of Foxp3 were analyzed for $CD8^+CD25^+$ T cells. Representative figures are shown. *B*, Intracellular Foxp3 expression was assessed in $CD8^+$ 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 $CD8^+CD25^+$ T cells after *D. pteronyssinus* 2-stimulated PBMCs were analyzed by flow cytometry. *D*, Increased expression of Foxp3 by purified $CD8^+CD25^+$ T cells was observed during IT by Western blot analysis. Ten independent experiments were performed, with essentially identical results.

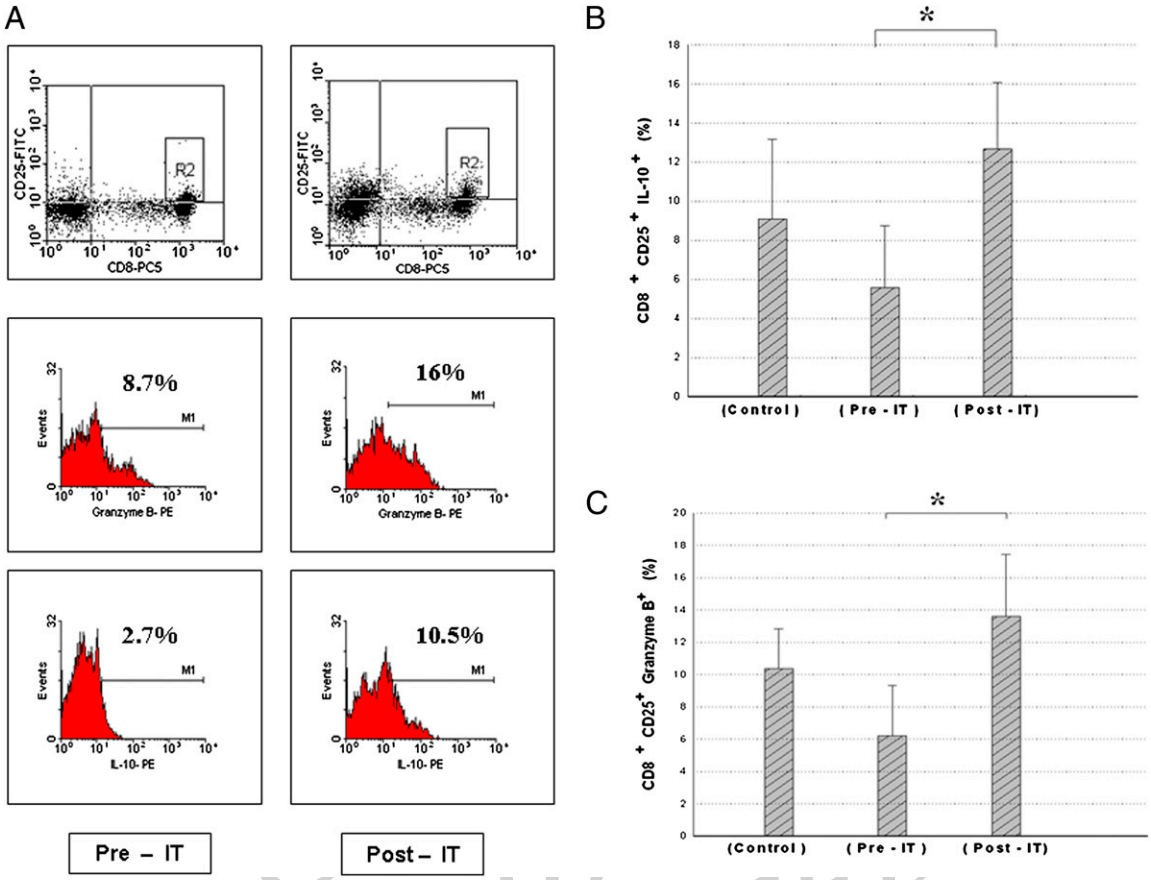

FIGURE 2. Intracellular IL-10 and granzyme B levels in $CD8^+CD25^+$ Treg cells before and after IT. PBMCs were stimulated with *D. pteronyssinus* 2 for 5 d, followed by stimulation with PMA (10 ng/ml) plus ionomycin (1 μg/ml) for the last 5 h and the addition of brefeldin A (10 μg/ml) for the final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

Costimulation with Pam3CSK4 and *D. pteronyssinus* 2 also significantly increased granzyme B expression in $CD8^+CD25^+$ T cells (19.29% ± 5.14% versus 9.33% ± 3.61%; $p < 0.05$) in nonatopic children (Fig. 3*D*).

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. 4*A*). Pam3CSK4 significantly suppressed *D. pteronyssinus* 2-induced IL-4 production by PBMCs from asthma patients and nonatopic controls (Fig. 4*A*). The production of IFN-γ, 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-γ production was markedly greater in Pam3CSK4-treated PBMCs compared with *D. pteronyssinus* 2-treated cells ($p < 0.05$; Fig. 4*B*). Pam3CSK4 stimulation elicited greater IL-10 cytokine production by PBMCs from asthma patients and nonatopic controls (Fig. 4*C*).

Suppressive activity of $CD8^+$ Treg cells involves cell contact

Next, experiments were performed to determine whether $CD8^+CD25^+$ Treg cells regulate $CD4^+$ T cell proliferation induced by Pam3CSK4 stimulation. PBMCs and $CD8^+$-depleted PBMCs from asthma patients before IT and nonatopic controls were stimulated with Pam3CSK4 and then labeled with CFSE. Purified, non–CFSE-labeled $CD8^+CD25^+$ Treg cells were added to $CD8^+$-depleted PBMCs, and cell proliferation was measured. In a representative study, $CD8^+CD25^+$ Treg cells suppressed the proliferation of $CD4^+$ T cells after stimulation with Pam3CSK4 (Fig. 5*A*). Fig. 5*B* summarizes the data from 20 asthmatic patients; $CD4^+$ T cell proliferation induced by anti-CD3 was enhanced following $CD8^+$ T cell depletion, and the addition of $CD8^+CD25^+$ Treg cells significantly inhibited proliferation. The suppression of $CD4^+$ T cell proliferation by $CD8^+CD25^+$ Treg cells was also observed in cells stimulated with Pam3CSK4 (Fig. 5*B*).

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 $CD8^+$ Treg cells and $CD8^+$-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 $CD4^+$ T cells were cocultured with $CD8^+$-depleted PBMCs (Fig. 5*C*). In summary, $CD8^+$ Treg cell-mediated suppression was largely dependent on cell contact.

$CD8^+CD25^+$ Treg cells involved in apoptosis of $CD4^+CD45RO^{hi+}$ cells

Our study probed $CD8^+CD25^+$ Treg cells triggering $CD4^+CD45RO^{hi+}$ cell apoptosis by *D. pteronyssinus* 2 during IT. The effect of $CD4^+$ and $CD8^+$ Treg cells on $CD4^+CD45RO^{hi+}$ apoptosis was assessed by determining the percentage of $TUNEL^+CD4^+CD45RO^{hi+}$ T cells in $CD25^+$-depleted PBMCs cocultured with purified $CD8^+CD25^+$ or $CD4^+CD25^+$ Treg cells. The percentage of $TUNEL^+CD4^+CD45RO^{hi+}$ T cells increased after the addition of $CD8^+CD25^+$ Treg cells (but not after the addition of

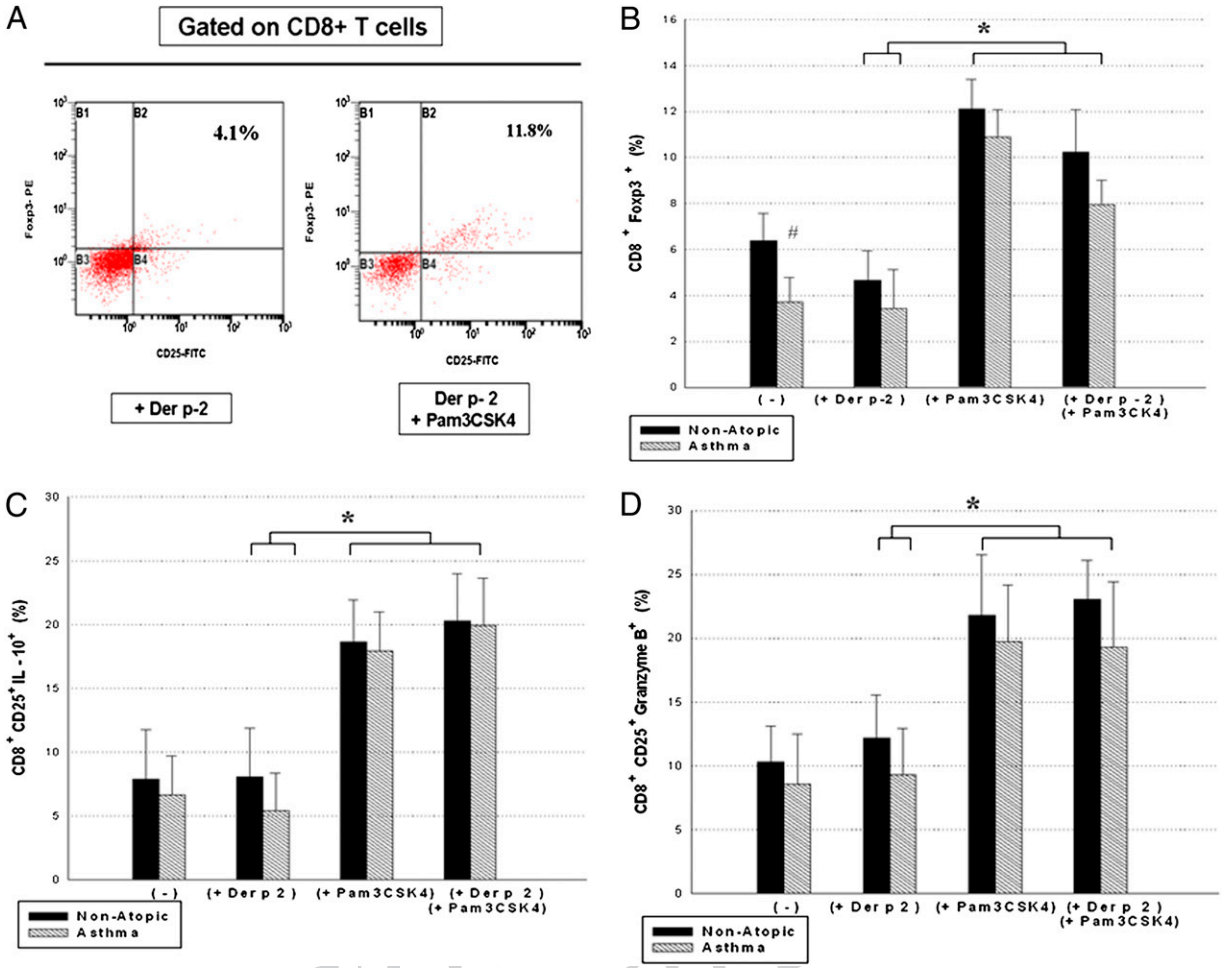

FIGURE 3. Foxp3 expression and intracellular IL-10 and granzyme B levels in $CD8^+CD25^+$ Treg cells stimulated with Pam3CSK4. PBMCs were stimulated with Pam3CSK4 and/or *D. pteronyssinus* 2 for 5 d. Summary of 30 paired experiments for intracellular Foxp3 expression in $CD8^+$ Treg cells (*A*) and $CD4^+$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

$CD4^+CD25^+$ Treg cells) to $CD25^+$-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 with supplement (Fig. 6).

Discussion

This study demonstrated that *D. pteronyssinus*-specific IT induced $CD8^+Foxp3^+$ Treg cells with increase of $CD8^+CD25^+IL\text{-}10^+$ and $CD8^+CD25^+$granzyme B^+ cells may partially account for $CD8^+$ Treg cell 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 $CD8^+CD25^+$ Treg cells to suppress T cell proliferation and increase $CD4^+CD45RO^+$ cell apoptosis. $CD8^+$ 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 $CD8^+$ Treg cells in peripheral blood, $CD8^+CD25^+Foxp3^+$ T cells can be generated by continuous Ag stimulation (12, 13). $CD8^+$ Treg cells were first identified in human tonsils; upon in vitro activation, $Foxp3^+CD8^+$ Treg cells were shown to inhibit T cell proliferation directly (12). $CD8^+$ 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 $CD8^+$ Treg cells that can inhibit the development of allergic diarrhea, suggesting that $CD8^+$ Treg cells may play a pivotal role in limiting allergic disease (33). In this study, we demonstrated that functional $CD8^+$ 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. *D. pteronyssinus* IT, by repeated Ag stimulation, may augment the $CD8^+$ 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-γ production and $CD8^+$ 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-β (36). Pam3CSK4 suppresses eosinophil infiltration in murine allergic conjunctivitis by inducing $CD4^+$ T cell apoptosis rather than by upregulating Th1 responses (37). Another anti-inflammatory

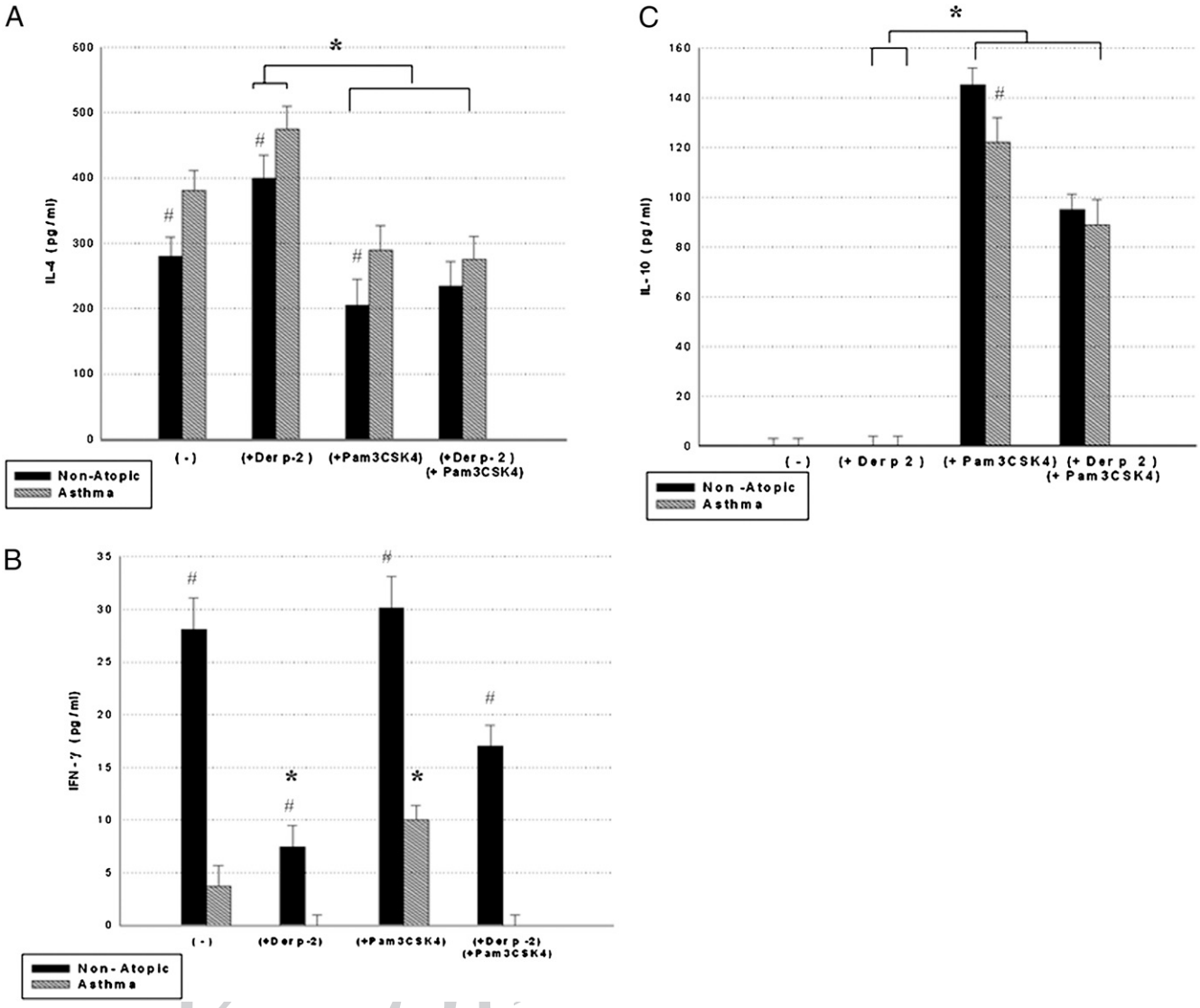

FIGURE 4. Production of IL-4 and -10 and IFN-γ by PBMCs costimulated with Pam3CSK4 and/or *D. pteronyssinus* 2. Supernatants from PBMCs stimulated with Pam3CSK4 and/or *D. pteronyssinus* 2 for 5 d were collected for analyses of IL-4 (*A*), IFN-γ (*B*), and IL-10 (*C*) production. #$p < 0.05$; asthmatic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

mechanism indicates that Pam3CSK4 directly activates $CD4^+CD25^+$ 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 $CD8^+Foxp3^+$ Treg cells to suppress $CD4^+$ 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 $CD8^+$ Treg cells, but not $CD4^+$ Treg cells, could enhance $CD4^+CD45RO^{hi+}$ cell apoptosis. Cell contact with $CD8^+$ Treg cells expressing increased granzyme B may induce cell apoptosis of $CD4^+CD45RO^+$ memory T cells during IT. In support of our findings, a functional study in autoimmune hepatitis subjects (38) revealed that $CD4^+CD25^{hi+}$ 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 $CD4^+CD25^+$ Treg cells are less able to suppress Th2 responses compared with Th1 responses (39–42). Grindebacke et al. (39) first demonstrated that allergen-stimulated $CD4^+$ Treg cells during IT failed to suppress Th2 responses, despite increased IL-10 production by T cells. Human thymus-derived $CD4^+CD25^+$ T cell clones suppress Th1 clone proliferation better than Th2 clone proliferation (40). The therapeutic transfer of $CD4^+CD25^+$ Treg cells only partially suppressed Th2-induced disease in an autoimmune gastritis model (41). Human purified $CD4^+CD25^{hi+}$ Treg cells isolated from PBMCs of control and cancer patients suppressed proliferation but did not mediate apoptosis in autologous $CD4^+CD25^-$ responder cells (42).

However, the interaction between the two subsets of Treg cells that protect against allergy remains unclear. Adoptive transfer of $CD4^+CD25^+$ 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 CD8 cells are required by $CD4^+CD25^+$ Treg cells, induced by a tolerogenic peptide, to suppress murine lupus (32). Therefore, $CD8^+$ Treg cells must cooperate with $CD4^+$ Treg cells after IT.

To conclude, our study illustrated that Pam3CSK4 ameliorates the Th2 allergic immune response by boosting $CD8^+$ Treg cell

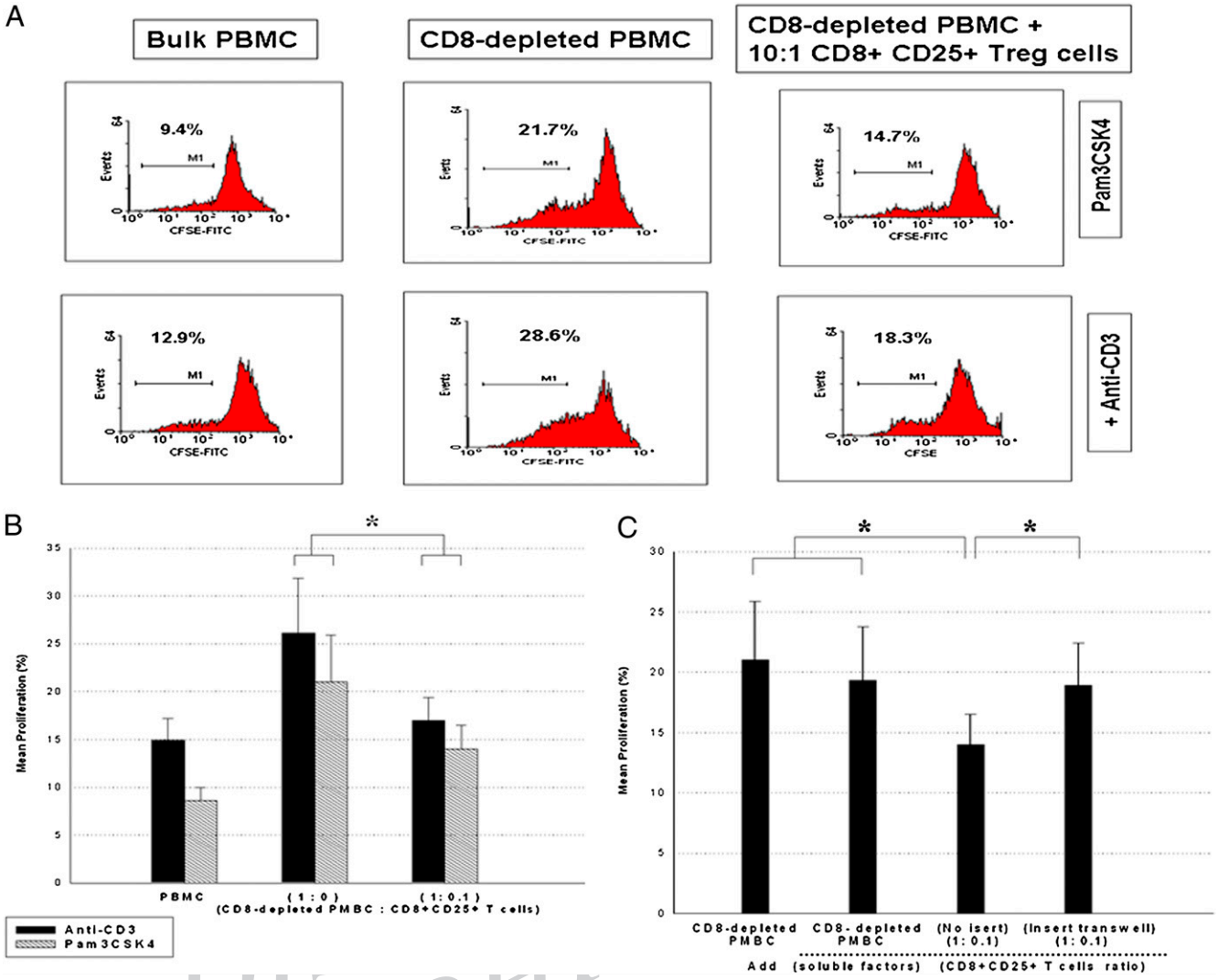

FIGURE 5. $CD4^+$ cell proliferation in the presence of $CD8^+CD25^+$ Treg cells. *A*, CFSE-labeled cells (bulk PBMCs and $CD8^+$-depleted PBMCs) were pretreated with anti-CD3 mAb or Pam3CSK4 for 5 d. The $CD8^+$-depleted PBMCs were incubated with purified $CD8^+CD25^+$ T cells at the ratio of 10:1. The proliferation of $CD4^+$ T cells was analyzed by flow cytometry. *B*, There was significant suppression (*) of $CD4^+$ cell proliferation in the presence of $CD8^+CD25^+$ Treg cells compared with $CD8^+$-depleted PBMCs alone. Data were calculated from 20 paired experiments. *C*, Requirement for cell–cell contact for $CD8^+$ Treg cell-mediated suppression. Autologous $CD8^+$-depleted PBMCs were cultured with supernatant of $CD8^+$ Treg cells and PBMCs or added to $CD8^+$ Treg cells at a ratio of 10:1 in the same well or separated by a Transwell semipermeable membrane. There was significant suppression (*) of $CD4^+$ T cell proliferation in the presence of cell–cell contact; this suppression did not occur without cell contact (proliferation similar to baseline).

function and decreasing Th2 cytokines. These findings further 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.

Table III. *Percentage of $TUNEL^+CD4^+CD45RO^{hi+}$ cells before and after the addition of Treg cells*

Group	$TUNEL^+CD4^+CD45RO^{hi+}$ (%; Mean ± SD)
Normal subjects	
$CD25^-$ PBMCs	5.72 ± 1.52
$CD25^-$ PBMCs and $CD4^+CD25^+$ Treg cells	6.32 ± 1.99
$CD25^-$ PBMCs and $CD8^+CD25^+$ Treg cells	10.28 ± 1.86*
Pre IT	
$CD25^-$ PBMCs	5.91 ± 2.05
$CD25^-$ PBMCs and $CD4^+CD25^+$ Treg cells	5.96 ± 2.35
$CD25^-$ PBMCs and $CD8^+CD25^+$ Treg cells	11.53 ± 3.54*
Post IT	
$CD25^-$ PBMCs	5.37 ± 2.17
$CD25^-$ PBMCs and $CD4^+CD25^+$ Treg cells	6.66 ± 3.30
$CD25^-$ PBMCs and $CD8^+CD25^+$ Treg cells	17.16 ± 4.27*,**

*$p < 0.05$, after the addition of Treg cells; **$p < 0.05$, among groups.

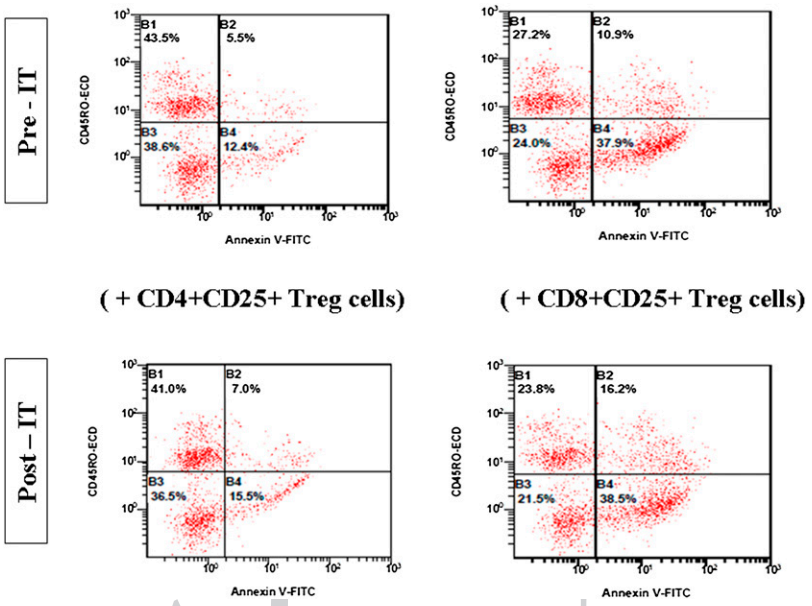

FIGURE 6. Double-fluorescence study by flow cytometry of CD45RO lymphocyte subpopulations and apoptosis during IT. $CD25^+$-depleted PBMCs were cocultured with $CD4^+CD25^+$ Treg cells or $CD8^+CD25^+$ Treg cells with *D. pteronyssinus* 2 stimulation for 5 d. Apoptosis in these cells was simultaneously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

Disclosures

Q:19 The authors have no financial conflicts of interest.

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