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Forkhead Box Protein 3 (FoxP3) Demethylation is Associated with Tolerance Induction in Peanut-Induced Intestinal Allergy

Meiqin Wang, MD, PhD[1], **Ivana V. Yang, PhD**[2,3], **Elizabeth J. Davidson, BS**[2], **Anthony Joetham, BS**[1], **Katsuyuki Takeda, MD, PhD**[1], **Brian P. O'Connor, PhD**[3], and **Erwin W. Gelfand, MD**[1]

[1]Department of Pediatrics, University of Colorado School of Medicine, Aurora, CO 80045

[2]Department of Medicine, University of Colorado School of Medicine, Aurora, CO 80045

[3]Center for Genes, Environment, National Jewish Health, Denver, CO 80206

Abstract

Background—Regulatory T cells (Tregs) play an essential role in the maintenance of immune homeostasis in allergic diseases.

Objectives—To define the mechanisms underlying induction of tolerance to peanut protein and prevention of the development of peanut allergy.

Methods—High or low doses of peanut extract (PE) were administered to pups every day for 2 weeks prior to peanut sensitization and challenge. Following challenge, symptoms, Treg numbers, FoxP3, Th2, Th17 cytokine and TGFβ expression in mesenteric lymph node (MLN) $CD4^+$ T cells and in jejunum were monitored. Treg suppressive activity and FoxP3 methylation in MLN $CD4^+$ T cells were assayed.

Results—Feeding high but not low doses of peanut prior to sensitization induced tolerance as demonstrated by prevention of diarrhea and peanut-specific IgE responses, increases in the percentage of $CD4^+CD25^+FoxP3^+$ cells in MLN, and FoxP3 mRNA and protein expression in $CD4^+$ cells from MLN or jejunum. Feeding high doses of peanut prior to sensitization decreased percentages of $CD3^+CD4^+IL13^+$ cells and $CD3^+CD4^+IL17^+$ cells in MLN and decreased *IL13, IL17A*, and increased *TGFβ* mRNA expression in the jejunum; numbers of $CD103^+$ DC in MLN were significantly increased. Treg suppression was shown to be antigen specific. FoxP3 methylation was increased in PE sensitized and challenged mice, whereas in tolerized mice, levels were significantly reduced.

Corresponding Author: Erwin W. Gelfand, MD, National Jewish Health, 1400 Jackson Street, Denver, CO 80206, Ph: 303-398-1196, Fax: 303-270-2105, gelfande@njhealth.org.

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Conclusions—Feeding high doses of peanut to pups induced tolerance to peanut protein. FoxP3 demethylation was associated with tolerance induction, indicating that Tregs play an important role in the regulation of peanut sensitivity and maintenance of immune homeostasis.

Keywords

DNA methylation; Treg cells; forkhead box protein 3; tolerance; peanut allergy

INTRODUCTION

Peanut allergy is a common and challenging food allergy in Western Europe and the United States (1–3). Development of food allergy is the result of the combination of genetic and environmental factors. Genetic variants in a number of genes have been associated with food allergy susceptibility using candidate gene approaches including filaggrin genes, HLA class II gene family (HLA-DRB1, HLA-DQB1, HLA-DPB1), forkhead box P3, and the NLR family (NLRP3), but results of association analyses have been inconsistent (4, 5). Clinical and experimental analyses suggest that initiation of food-induced intestinal allergy is regulated by numerous inflammatory cells and mediators (6–8). Recently peanut oral and epicutaneous immunotherapy clinical trials have been performed in peanut allergic children (9, 10). However, mechanistic studies of food allergy are necessarily limited as target organ patient specimens such as gut tissue (biopsy) are often difficult to obtain on a regular basis. We investigated a well-characterized mouse model of peanut sensitization to manipulate and define the mechanisms underlying the induction of oral tolerance to peanut (6, 7).

Oral tolerance is a state of antigen-specific systemic hyporesponsiveness or unresponsiveness to an antigen to which an individual or animal has been previously exposed via the oral route (11). Oral tolerance has been well studied in autoimmune diseases and has been used in clinical settings for treating autoimmune patients such as rheumatoid arthritis (12) and systemic sclerosis (13). A number of clinical trials are underway examining the efficacy of oral or transcutaneous tolerance induction to peanut. However, the molecular basis underlying development of oral tolerance in food allergy remains to be defined, in particular the role of epigenetic mechanisms. Regulatory T cells (Tregs) have been shown to control a number of allergic diseases including allergic asthma and atopic dermatitis (14, 15). Here, the role of Tregs in the development of oral tolerance to peanut protein was investigated. We demonstrated that Tregs played a critical role in the induction of oral tolerance to peanut protein and that FoxP3 demethylation was involved in this process.

MATERIALS AND METHODS

Mice

Pups were derived from ten- to twenty-week-old BALB/c wild-type (WT) mice, which were purchased from the Jackson Laboratory (Bar Harbor, ME). All mice were maintained on a peanut- and soy-free diet under specific pathogen-free conditions. All studies were conducted under a protocol approved by the Institutional Animal Care and Use Committee of National Jewish Health (Denver, CO).

Preparation of peanut protein

Crude peanut extract (PE) was prepared from defatted raw flours (Golden Peanut Company, Alpharetta, GA) as previously described (16). Endotoxin levels in PE solutions were less than 0.1 EU/ml as assessed by a Chromogenic LAL endotoxin assay kit (GeneScript, Piscataway, NJ).

Induction of tolerance to peanut- induced intestinal inflammation in vivo

Naive, 7 day old pups, received daily PE at different doses (0.05 or 0.5 mg/gram body weight) by gavage using a 24-gauge feeding needle (Fisher Scientific, Pittsburgh, PA) in a total of volume of 20 μl of PBS for 2 weeks. The doses were chosen based on initial experiments investigating induction of tolerance. Two weeks later the mice were sensitized and challenged with PE (PE0.05/PE/PE, PE0.5/PE/PE) as previously described (16). Briefly, mice were sensitized 3 times with 500 μg of PE together with 2.0 mg of alum (Pierce) by intraperitoneal (i.p.) injection in a total volume of 100 μL on days 1, 7, and 21. Two weeks later, mice received 20 mg of PE (in 250 μL of PBS) by gavage with a 22-gauge feeding needle (Fisher Scientific) every day for 1 week. Twenty-four hours after the last challenge, serum, mesenteric lymph nodes (MLN), and jejunal tissues were collected and analyzed. Control animals were sham-fed and sham-sensitized and PE challenged mice (PBS/PBS/PE) or sham-fed and PE sensitized and challenged mice (PBS/PE/PE).

Assessment of hypersensitivity reactions

Allergic symptoms were evaluated 30 minutes every day after the oral challenge, as previously reported (17) as follows: 0, no symptoms; 1, scratching and rubbing around the nose and head; 2, puffiness around the eyes and mouth, diarrhea, pilar erecti, reduced activity, and/or decreased activity with increased respiratory rate; 3, wheezing, labored respiration, and cyanosis around the mouth and tail; 4, no activity after prodding or tremor and convulsion; and 5, death. Scoring of symptoms was performed in a blinded manner by an independent observer.

Histology

Jejunal tissue was fixed in 10% formalin and processed into paraffin blocks. Jejunal tissue expression of FoxP3 was identified by immunohistochemistry (IHC) staining using anti-mouse FoxP3 antibody (Abcam, Cambridge, Mass). Quantification of stained FoxP3-positive cells per square millimeter of lamina propria was performed with an Olympus microscope linked to the National Institutes of Health Image Analysis Program (NIH, Bethesda, MD).

Anti-CD25 antibody production and purification

Rat anti-CD25 (IL-2Ra) monoclonal antibody was isolated from the PC61 hybridoma (kindly provided by Dr. Ross Kedl, University of Colorado, Aurora, CO) originally obtained from the American Type Culture Collection (ATCC, Manassas, VA) as previously described (18). Protein concentrations were determined from the optical density at 280 nm. Endotoxin levels in purified antibody clones PC61 and control rat IgG (clone HRPN) were less than 0.1

EU/ml, as assessed by using a Chromogenic LAL endotoxin assay kit (GeneScript, Piscataway, NJ).

In vivo depletion of regulatory T cells

In vivo depletion of $CD4^+CD25^+$ Tregs was achieved following i.p. injection of 500 μg of monoclonal anti-CD25 antibody (Clone PC61) on days −21, −1, and 20 (3 times prior to sensitization or during sensitization on days stated in the experiment, Fig. 4A). Control mice were injected with 500 μg of rat IgG on the same days as anti-CD25. The doses of anti-CD25 were chosen based on previous reports (19, 20). Depletion of Treg cells was confirmed by flow cytometry following staining of peripheral blood and spleen lymphocytes with anti-CD4 and anti-CD25 (7D4), one day after injection of the anti-CD25 antibody.

Measurement of peanut-specific antibody

Serum peanut-specific IgE, IgG1, and IgG2a levels were measured by ELISA as described previously (17, 21).

Isolation of mononuclear cells from the mesenteric lymph nodes

MLN mononuclear cells from BALB/c mice were isolated using a plunger and cell suspensions were passed through a Falcon cell strainer (70-μm mesh, BD Biosciences, San Jose, CA).

Flow cytometry

Cells from MLN or peripheral blood were labeled with anti-CD4 and anti-CD25 antibodies and stained for intracytoplasmic FoxP3 using an anti-mouse FoxP3 intracellular staining kit (eBiosciences) according to the manufacturer's protocol. Cells from MLN were labeled with anti-CD3 and anti-CD4 antibodies and stained for intracytoplasmic IL-13, IL-17A, IFN-γ, and IL-10. To identify DC subsets, cells from MLN were labeled with anti-CD11c, anti-MHCII, anti-CD11b, anti-CD103, and anti-B220 antibodies, as well as 4', 6-diamidino-2-phenylindole (DAPI). The populations were gated on DAPI negative and B220 negative cells. The $CD11c^+$MHCIIhigh population was further characterized on the basis of CD103 and CD11b expression. All antibodies were obtained from BD Biosciences, eBiosciences, or BioLegend (San Diego, CA). Cells were analyzed on a FACSCalibur (BD Biosciences) using FlowJo software (Tree Star, Ashland, OR).

Assessment of Treg function

Mononuclear cells (MNC) were isolated from spleen of peanut and ovalbumin (OVA)-immunized mice by density gradient centrifugation as previously described (22). MNC were labeled with 5 μM of carboxyfluorescein succinimidyl ester (CFSE) (eBioscience) according to the manufacturer's directions. $CD4^+CD25^+$ T regulatory cells were isolated from the mesenteric lymph nodes of tolerized WT mice (PE0.5/PE/PE) by sorting (MoFlo XDP, Beckman Coulter) with a purity of more than 95%. Isolated $CD4^+CD25^+$ Tregs were co-cultured with peanut or OVA and CFSE-labeled MNC. The doses of OVA 10 μg/ml and PE 200 μg/ml were chosen based on our previous report (22) and initial titration of PE

concentrations, respectively. Following 5 days co-culture, numbers of divisions of CFSE-labeled MNC were monitored by flow cytometry.

Purification of CD4$^+$ T cells and quantitative RT-PCR

$CD4^+$ T cells were isolated from BALB/c WT mouse mesenteric lymph nodes (MoFlo XDP, Beckman Coulter) with a purity of more than 98% as determined by flow cytometry. RNA was extracted from freshly sorted MLN $CD4^+$ T cells or jejunal tissue homogenates using Trizol (Invitrogen, Carlsbad, CA). cDNA was generated with the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Quantitative RT- PCR was performed on the ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA). All primers and probes used were purchased as TaqMan Gene Expression Assays from Applied Biosystems. Fold changes were calculated by using the delta-delta cycle threshold ($\Delta\Delta C_T$) method.

DNA bisulfite and pyrosequencing

$CD4^+$ T cells were isolated from MLN of BALB/c WT mice as described above. Genomic DNA from $CD4^+$ T cells was extracted using DNeasy blood kit (Qiagen, Valencia, CA) and quantified with a spectrophotometer. DNA (300 ng) from $CD4^+$ T cells was bisulfite-converted using EpiTect fast DNA bisulfite kit (Qiagen). Pyrosequencing of FoxP3 methylation at the Treg-specific demethylation region (TSDR) in isolated $CD4^+$ T cells was performed (assay-ID: ADS568FS1, ADS568FS2, EpigenDx) using the Pyromark Q96 MD (Qiagen) according to manufacture's directions. The CpG site locations were: CpG site # −27 (−2369 from ATG), −26 (−2353 from ATG), −25 (−2319 from ATG), −24 (−2292 from ATG), −23 (−2287 from ATG), −22 (−2238 from ATG), −21 (−2219 from ATG), −20 (−2215 from ATG), and −19 (−2207 from ATG).

Statistical analysis

ANOVA was used to evaluate differences among experimental groups. Pairwise comparisons between groups utilized the post-hoc Tukey-Kramer highest significance difference test. P values for significance were set at 0.05. All results were expressed as the means±SEM.

RESULTS

Peanut feeding in neonates suppresses peanut allergic responses

We sought to develop a means for preventing peanut-induced intestinal inflammation through the induction of oral tolerance. Beginning at 7 days of age, pups received peanut, 0.05 or 0.5 mg/ gram body weight, for 2 weeks before sensitization followed by peanut sensitization and challenge. The protocol to induce oral tolerance to peanut protein and the experimental groups are indicated in Figure 1A. Feeding low doses of PE prior to sensitization and challenge (PE0.05/PE/PE) failed to impact development of diarrhea, as shown in PE sensitized and challenged mice (PBS/PE/PE) (Fig. 1B). In contrast, feeding high doses of PE to the pups prior to sensitization and challenge (PE0.5/PE/PE) induced tolerance to peanut protein and prevented development of diarrhea (Fig. 1B). PE0.05/PE/PE and PBS/PE/PE mice showed higher symptom scores (Fig. 1C) and higher levels of serum PE-specific IgE, IgG1, and IgG2a (Fig. 1D) compared to controls (PBS/PBS/PE). Feeding

high doses of PE to PE sensitized and challenged mice significantly reduced symptom scores (Fig. 1C) and levels of serum PE-specific IgE, IgG1, and IgG2a compared to the PE0.05/PE/PE and PBS/PE/PE groups (Fig. 1D). Together, these results demonstrated that feeding high doses of PE to neonates prior to sensitization induced tolerance to peanut protein, whereas feeding low doses of PE failed to do so.

Early feeding of peanut alters the number of Tregs and FoxP3 expression in MLN CD4$^+$ T cells and jejunum

Tregs are involved in allergic sensitization to foods and modify the adaptive immune response but mechanisms remain unclear (23). We monitored numbers of Tregs and the expression of the Treg-associated gene FoxP3 in MLN CD4$^+$ T cells and in the jejunum by flow cytometry, RT-PCR, and IHC. We demonstrated a 10-fold reduction in *FoxP3* mRNA expression in MLN CD4$^+$ T cells from PE sensitized and challenged mice compared with controls (Fig. 2A). Feeding high but not low doses of peanut prior to sensitization significantly increased *FoxP3* mRNA expression in MLN CD4$^+$ T cells (Fig. 2A). FoxP3 protein was localized to the lamina propria of jejunal tissues and higher numbers of FoxP3$^+$ cells were seen in the lamina propria of jejunal tissue in PE0.5/PE/PE mice compared with PBS/PE/PE and PE0.05/PE/PE mice (Figs. 2B, 2C). The percentages of CD4$^+$CD25$^+$FoxP3$^+$ in MLN were decreased in PBS/PE/PE mice (30±1.1%) compared with control PBS/PBS/PE mice (43±1.5%) (Fig. 2D). Feeding high doses of PE prior to sensitization markedly increased the percentages of CD4$^+$CD25$^+$FoxP3$^+$ cells in MLN (63±2.1%) compared with control PBS/PE/PE mice (Fig. 2D). However, feeding low doses of PE failed to increase the percentages of CD4$^+$CD25$^+$FoxP3$^+$ cells in MLN (36±1.2%). These data indicated that numbers of Tregs and FoxP3 expression were associated with induction of oral tolerance to peanut protein.

Early feeding of peanut alters cytokine and transcription factor expression in MLN CD4$^+$ T cells and jejunum

A number of specific cytokines have been implicated in the development of allergic disorders including food allergy and asthma (7, 24). We measured levels of cytokine and transcription factor mRNA expression in jejunal tissue. Sham feeding and PE sensitization and challenge resulted in increased *IL13* and *IL17A* and decreased *TGFβ* mRNA expression without effects on *IFNG and IL10* mRNA expression in jejunal tissue compared to controls (PBS/PBS/PE) (Fig. 3A). Feeding high doses but not low doses of PE prior to sensitization decreased *IL13 and IL17A* and increased *TGFβ* mRNA expression*; IFNG and IL10* mRNA were not altered compared to controls (PBS/PE/PE) (Fig. 3A). In parallel, expression of the lineage-specific transcription factors *GATA3* and *RORγt* mRNA in jejunal tissue was significantly decreased in mice fed high doses of PE compared to control (PBS/PE/PE) mice; levels of expression of *T-bet* mRNA were not altered in mice fed high doses of PE compared to control (PBS/PE/PE) mice (Fig. 3B).

Similarly, feeding high doses but not low doses of PE prior to sensitization decreased the percentages of CD3$^+$CD4$^+$IL-13$^+$ (from 1.91±0.16% to 0.8±0.1%) and CD3$^+$CD4$^+$IL-17A$^+$ cells (from 1.03±0.08% to 0.64±0.09%) in MLN compared to control (PBS/PE/PE) mice (Fig. 3C). The percentages of CD3$^+$CD4$^+$IL-10$^+$ cells in MLN also were increased (from

0.89±0.11% to 1.13±0.12%) but did not reach statistical significance in mice fed high doses of PE compared to control (PBS/PE/PE) mice (Fig. 3C). The percentages of $CD3^+CD4^+IFN\gamma^+$ cells in MLN were similar in all groups.

Tolerance induction induces an increase in the number of CD11c+MHCII^{high} CD103$^+$CD11b$^-$ DC

DCs play a central role linking the innate and adaptive immune systems and orchestrating immune responses. Following peanut sensitization and challenge, increases in the percentage of migratory DC ($CD11c^+MHCII^{high}$) from 0.35 ± 0.03% to 0.91 ± 0.01% and decreases in the percentage of $CD11c^+MHCII^{high}CD103^+CD11b^-$ DC (named $CD103^+$DC) from 9.91 ± 0.23% to 6.22 ± 0.16% in MLN were detected (Fig. 4, and see Fig E1 in this article's Online Repository at www.jacionline.org). Feeding high but not low doses of peanut prior to sensitization markedly decreased the percentages of migratory DC ($CD11c^+MHCII^{high}$) (from 0.91 ± 0.01% to 0.55 ± 0.03%) and increases in $CD103^+$DC (from 6.22 ± 0.16% to 9.31 ± 0.46%) in MLN compared with control PBS/PE/PE mice (Fig. 4, and see Fig El in this article's Online Repository at www.jacionline.org). The percentages of $CD11c^+MHCII^{high}CD103^+CD11b^+$ and $CD11c^+MHCII^{high}CD103^-CD11b^+$ DC subsets did not change. These data indicated that migratory DC and $CD103^+$DC were associated with induction of oral tolerance to peanut protein.

Antigen-specific suppression of CD4$^+$CD25$^+$ cells

To determine whether Treg suppressive function was antigen specific, a functional assay was carried out in vitro. Spleen MNC from PE-sensitized BALB/c mice proliferated in response to PE in a dose-dependent manner; based on initial studies, the optimal dose of 200 μg/ml PE was chosen. PE-induced proliferation of MNC was markedly decreased in the presence of $CD4^+CD25^+$ cells from peanut tolerant mice compared to controls (in the absence of $CD4^+CD25^+$ cells) (from 41.5 ± 0.9% to 21.8 ± 2.1%, $P<0.05$) (Figure 5). In contrast, OVA-induced proliferation of MNC was only marginally decreased in the presence of $CD4^+CD25^+$ cells from peanut tolerant mice compared to controls (in the absence of $CD4^+CD25^+$ cells) (from 40.5 ± 2.6% to 39 ± 1.7%, $P>0.05$) (Figure 5). These data indicated that Treg suppressive activity was antigen specific.

Treatment with anti-CD25 attenuates oral tolerance to peanut protein

Given the link between Treg numbers and FoxP3 expression with development of oral tolerance, we determined the consequences of reducing Treg numbers by administering anti-CD25 prior to and during the sensitization phase. Peanut tolerant WT mice (PE0.5/PE/PE) were treated with anti-mouse CD25 by i.p. injection 3 times before and during sensitization (on days −21, −1, and 20) (Fig. 6A). The doses used were based on previous studies effectively depleting Tregs (19, 20). Peanut tolerant mice treated with anti-CD25 had an increase in diarrhea, and significantly increased clinical symptom scores compared with peanut tolerant mice treated with control antibody (Figs. 6B, 6C). Serum levels of peanut-specific IgE, IgG1, and IgG2a were also increased in peanut tolerant mice treated with anti-CD25 compared with tolerant mice treated with control antibody (Fig. 6D). After treatment with anti-CD25, the percentages of $CD4^+CD25^+FoxP3^+$ cells in MLN following feeding of high doses of PE were significantly decreased (37±1.5%, similar to PBS/PE/PE mice)

compared with mice treated with control antibody (65±1.2%) (Fig. 6E). Anti-CD25 administration depleted $CD4^+CD25^+$ T cells from both spleen and peripheral blood. $CD4^+CD25^+$ T cells by approximately 20-fold (from 2.29 to 0.12%) and 10-fold (from 5.17 to 0.51%) from spleen and peripheral blood, respectively, detected 1 day after the second injection of anti-CD25 (Fig. 6F). Peanut tolerant mice treated with anti-CD25 increased *IL13* and *IL17A* and decreased *TGFβ* mRNA expression without impairing *IFNG and IL10* mRNA expression in jejunal tissue compared with tolerant mice treated with control IgG (Fig. 6G). In parallel, *GATA3* and *RORγt* mRNA in the tissues were significantly increased in tolerant mice treated with anti-CD25. Levels of *T-bet* mRNA expression were not altered in tolerant mice treated with anti-CD25 (Fig. 6H). Together, these data demonstrated that peanut tolerant mice treated with anti-CD25 reversed the ability to be tolerized with reappearance of all clinical, cellular, and molecular manifestations of peanut sensitization, indicating that Tregs played an important role in the regulation of intestinal allergic responses and maintenance of tolerance to a food allergen.

FoxP3 DNA demethylation is associated with induction of tolerance

To investigate the involvement of epigenetic mechanisms in development of oral tolerance to peanut protein, we assessed FoxP3 methylation in genomic DNA from MLN $CD4^+$ T cells at individual CpG sites by bisulfite pyrosequencing. Since FoxP3 demethylation at CNS2 is required for stable FoxP3 expression (25–27), we targeted CpG sites at CNS2 of the first intron of the FoxP3 gene (Fig. E2 in the online supplement) which included 9 CpG sites in a Treg-specific DNA demethylation region. We found significantly increased FoxP3 methylation at number −27, −26, −24, −22, −21, −20, and −19 (−2369, −2353, −2292, −2238, −2219, −2215, −2207 from ATG, respectively) positions of the 9 CpG sites within the intronic 1 region in sham-fed and PE sensitized and challenged mice compared with sham-fed and sham-sensitized and PE challenged controls (Fig. 7). In parallel to induction of tolerance, levels of FoxP3 methylation in 7 of the 9 CpG sites in the intronic 1 region were significantly reduced, almost to baseline levels (Fig. 7). Levels of FoxP3 methylation were not altered in mice fed low doses of PE prior to sensitization and challenge except for CpG positions −22 and −19 compared to sham-fed and PE sensitized and challenged mice (Fig. 7). FoxP3 methylation at the −25 position site was unchanged among the 4 groups. Measurements of FoxP3 methylation at the −23 position failed due to high CpG sum deviation. These data indicated FoxP3 demethylation at CNS2 was associated with the induction of tolerance to peanut protein.

DISCUSSION

Oral tolerance refers to a state of local and systemic immune unresponsiveness that is induced by oral administration of soluble antigens such as food proteins. It has been applied therapeutically to prevent or treat a number of immune–mediated diseases including allergic disorders, in particular food allergy (23), and autoimmune diseases (28). To induce tolerance, targeting the adaptive immune response and antigen-specific responses is required. The lamina propria, the gut-associated lymphoid tissues, such as Peyer’s patches, and the gut-draining mesenteric lymph nodes are the major sites of antigen uptake by dendritic cells that encounter and activate naive T cells in the MLN (11). The MLN cells are

the initiators of oral tolerance induction and are considered critical to the process (11, 29). We established oral tolerance to peanut protein in an experimental model of peanut allergy in order to directly define events in the gastrointestinal tract and MLN. We found that feeding high but not low doses of peanut to neonatal mice prior to peanut sensitization and challenge induced a state of tolerance to peanut protein. These data demonstrated that tolerance to peanut can be effectively induced but that tolerance induction is dependent on the amount of antigen fed. Translating such an animal study to humans is clearly difficult. Nevertheless, based on this mouse study, it is estimated that if infants at 7 or 8 months of age introduced peanut, about 0.3 gram per day in the diet, beneficial desensitization to peanut protein may ensue (30, 31). The timing of initial feeding and the amount of allergen fed may be critical determinants for inducing tolerance in infants (32). Recent clinical studies have emerged demonstrating the effectiveness of early feeding of peanut to non-sensitized infants which decreased the development of peanut allergy over the ensuing 4–5 years (33). Tolerance lasted for the one year children were monitored following cessation of peanut ingestion (34), but the molecular and epigenetic mechanisms underlying the benefits remain to be clarified.

Multiple immune cells and mediators are implicated in the pathogenesis of food allergy (6, 35–37). A critical subset include populations of Tregs which are involved in the suppression of immune responses and maintaining immune homeostasis. Although there are several forms of regulatory T cells, $FoxP3^+$ naturally occurring Tregs (nTregs) and $FoxP3^+$ inducible Tregs (iTregs) are two well-defined subsets (38). nTregs are selected in the thymus as a consequence of their reactivity to self antigens whereas iTregs are generated from naive CD4 T cells in the peripheral immune system and can differentiate into other helper T cells under inflammatory conditions (39, 40). FoxP3 is a specific marker of T regulatory cells and serves as a lineage specification transcriptional factor of Tregs. In both mice and humans, mutations of *FoxP3* result in a complex syndrome of immune dysregulation and enteropathy (41, 42). Acquired states of FoxP3 deficiency leading to loss of Treg function have also been associated with allergic diseases such as asthma (43). In mice, depletion of $FoxP3^+$ Tregs resulted in autoimmune inflammation and colitis (44). Taken together, the data point to the central role of Tregs in controlling allergic and autoimmune manifestations in different target organs.

In the present study, we focused on T cell populations present in the jejunal tissue and in the MLN, the primary inductive sites for oral tolerance; tolerogenic potential is largely confined to the MLN (29). We demonstrated that tolerance induction was associated with increases in numbers of $CD4^+CD25^+FoxP3^+$ cells in the MLN, and FoxP3 mRNA and protein expression in MLN $CD4^+$ T cells and in the jejunum. It has been suggested that low doses of antigen favor generation of Tregs, whereas higher doses tend to favor induction of anergy or deletion (45). Antigens such as peanut protein are acquired in the lamina propria of the small intestine and are carried to the mesenteric lymph node by $CD103^+$ DCs (23). $CD103^+$ DCs induce the development of inducible Treg (iTreg) cells, which suppress pro-allergic Th2 responses and regulate tolerance. In this study we observed increases in numbers of Tregs in the MLN as well as increases in numbers of $CD103^+$ DCs following feeding of high-dose peanut. In contrast, lower numbers of MLN $CD103^+$ DCs were seen following feeding of low doses of peanut. Since $CD103^+$ DCs play an important role in maintenance of intestinal

homeostasis through iTreg cell generation (46), the lower numbers of $CD103^+$ DCs following low dose feeding may account, in part, for the lower number of Tregs and failure of tolerance induction. These data indicate that Tregs expressing FoxP3 can attenuate allergic responses to food antigens in sensitized mice and the findings are consistent with reports of oral tolerance induced following a high dose feeding regimen (29).

To confirm the role of $FoxP3^+$ Tregs, Tregs were depleted following administration of anti-CD25 prior to and during the sensitization phase. Depletion of Tregs was associated with a loss of tolerance and appearance of all manifestations of peanut allergy. In a similar manner depletion of Tregs enhanced development of autoimmune disease (47) and, by contrast, adoptive transfer of $CD4^+CD25^+$ T cells into CD4 T cell- deficient mice enabled oral tolerance induction in a model of contact hypersensitivity (48). Interestingly, oral tolerance to antigen could be induced by iTregs but not nTregs, implying *in vivo* peripheral conversion of naive $CD4^+$ T cells into iTregs (14). We have recently shown that both nTregs and iTregs can suppress lung inflammation in an experimental model of asthma, and that both populations of Tregs have the capacity to convert into potent effector cells through different cytokine-driven pathways (49).

Cytokines play an important role in antigen-induced adaptive immune responses in food allergic models (16). In the present study, we demonstrated that with tolerance induction, decreased percentages of $IL\text{-}13^+$ and $IL\text{-}17^+$ MLN $CD4^+$ T cells were found without alterations in IFN-γ^+ or $IL\text{-}10^+CD4^+$ T cells. Tolerant mice showed decreased *IL13* and *IL17A* mRNA expression and increased *TGFβ* mRNA expression in the jejunum without altering *IFNG* or *IL10* expression. These data indicated that the cytokine changes associated with the allergic responses in the jejunum were effectively attenuated with tolerance induction. TGF-β, retinoic acid, and microbial antigens promote differentiation and expansion of peripheral Tregs from naive $CD4^+$ T cells (50). TGF-β is a key cytokine in the generation of $FoxP3^+$ Tregs in MLN (51). Our results showed that oral tolerance induction was accompanied by enhanced TGF-β responses but not IL-10, which are consistent with the findings of Mucida et al. demonstrating that oral-induced tolerance is dependent on TGF-β but not IL-10 (52). Nonetheless, there are conflicting data on the role of IL-10 in Treg-mediated immune suppression and tolerance, perhaps in part due to the models used and dose-dependent effects of IL-10 (53).

Epigenetic modifications such as DNA methylation, histone acetylation, and chromatin remodeling of critical gene loci are involved in allergic airway disease and food allergy (54–56). The stability of FoxP3 expression in Tregs is controlled by DNA methylation (57). Transcriptional silencing of FoxP3 via hypermethylation of CpG islands in the promoter and intronic regions has been identified in patients with allergic disease, including asthma (58, 59) and has been associated with reduced Treg function. By measuring demethylation of TSDR in FoxP3, prenatal modulation of Treg numbers was suggested (60). FoxP3 hypermethylation in peripheral blood DNA was associated with diminished Treg function and increased asthma severity in children exposed to polycyclic aromatic hydrocarbons (59). In a trial of oral immunotherapy to peanut, “resensitization” was associated with increased methylation of CpG in the FoxP3 locus of isolated Tregs (35). It is interesting to note that farm milk consumption, which has been associated with asthma-protective effects, was

associated with higher FoxP3 demethylation and higher Treg cell numbers (61), as seen here in the PE tolerant mice. These studies and our results suggest that tolerance induction to food protein is associated with epigenetic modification, DNA methylation, of FoxP3.

Conserved non-coding DNA sequence (CNS) elements at the *FoxP3* TSDR locus encode information defining the size, composition, and stability of Treg cells (25). CNS2, although dispensible for induction of *FoxP3* (25), was required for *FoxP3* expression in the progeny of dividing Tregs (25, 26) and CNS2 deficiency markedly impaired *FoxP3* expression (25, 26). FoxP3 binds to CNS2 in a CpG DNA demethylation-dependent manner, conferring Treg lineage stability; both demethylation of the CpG island in CNS2 and FoxP3 binding to CNS2 were associated with stable expression of *FoxP3* (25, 27, 62). We analyzed FoxP3 methylation at the intronic 1 region of CNS2 by bisulphite pyrosequencing. We found that tolerance to peanut protein was associated with FoxP3 demethylation in MLN $CD4^+$ T cells. This suggests that tolerance induction was involved in or associated with epigenetic regulation of FoxP3 methylation. Although FoxP3 demethylation was shown in MLN $CD4^+$ T cells, and not isolated Tregs, FoxP3 demethylation of TSDR, a factor promoting or stabilizing FoxP3 gene transcription and expression, was restricted to Tregs. To directly link the changes in and extent of FoxP3 methylation as well as differential methylation at individual CpG sites within the TSDR with antigen-specific inducible Treg function and tolerance induction will require further study. Taken together, the studies identify epigenetic regulation of T regulatory cell function as a potential target for tolerance induction and an opportunity for translational studies in man (63).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

CNS	Conserved non-coding DNA sequence
CFSE	Carboxyfluorescein succinimidyl ester
DAPI	4’, 6-diamidino-2-phenylindole
DC	Dendritic cell
FoxP3	Forkhead box protein 3
IHC	Immunohistochemistry
i.p.	Intraperitoneal

MHC II	MHC class II
MNC	Mononuclear cell
MLN	Mesenteric lymph node
iTregs	Inducible Tregs
nTregs	Naturally occurring Tregs
OVA	Ovalbumin
PE	Peanut extract
RORγt	Retinoic acid-related orphan receptor γt
RT-PCR	Real-time polymerase chain reaction
Tregs	Regulatory T cells
TSDR	Treg-specific demethylation region
WT	Wild-type

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Clinical Implications

The state of Foxp3 demethylation in MLN $CD4^+$ T regulatory cells is associated with induction of tolerance to peanut protein.

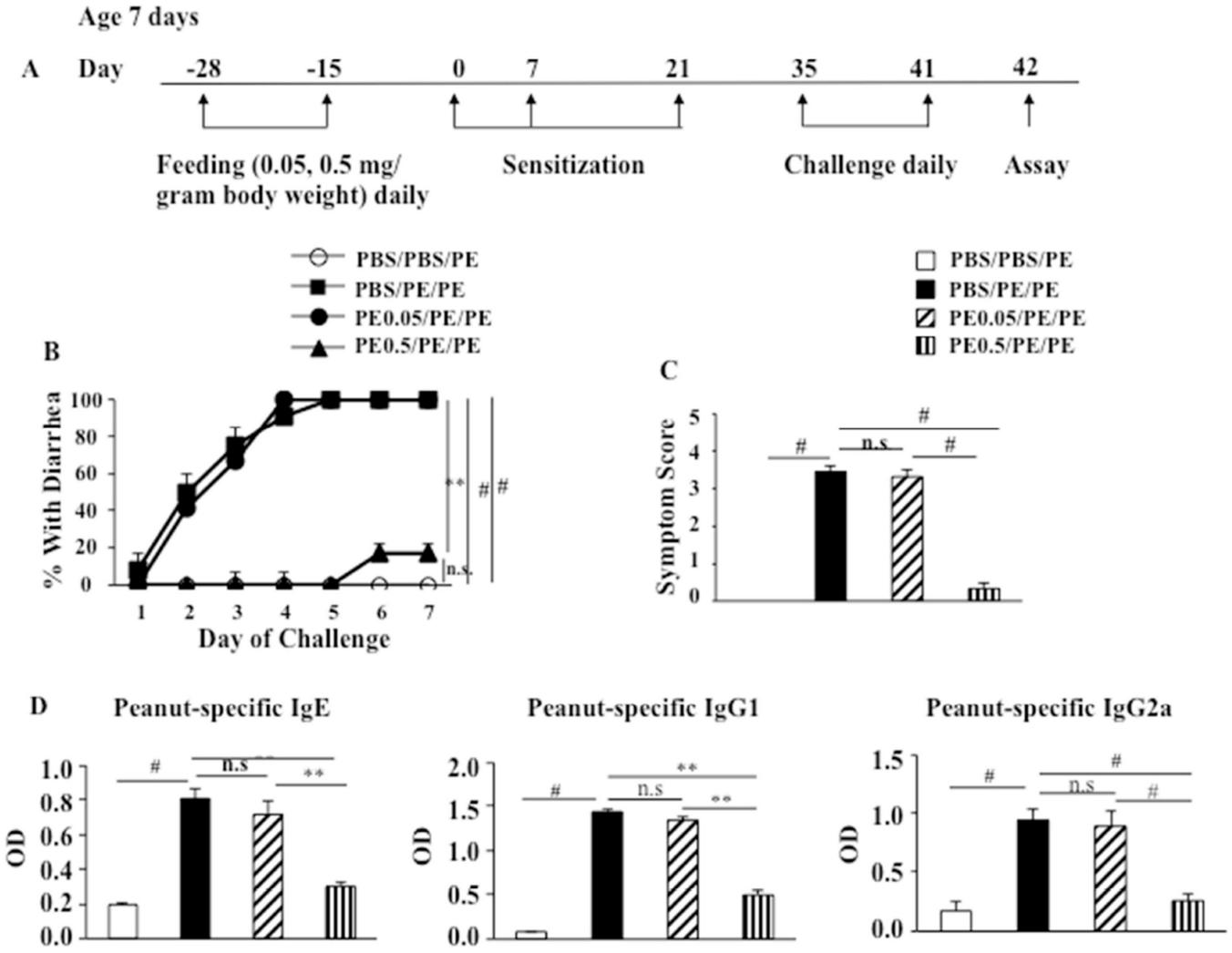


Figure 1.

Oral PE administration in pups prior to sensitization induces tolerance to peanut protein. (A) Protocol for induction of peanut allergy and induction of tolerance to peanut protein. PE was fed to pups from day −28 to day −15. Sensitization was performed on days 1, 7, and 21. Challenge was performed from day 35 to day 41. (B) Kinetics of the development of diarrhea after feeding PE prior to sensitization. (C) Scores based on the severity of clinical signs were assessed 30 minutes after oral challenge. (D) Serum levels of peanut-specific IgE, IgG1, and IgG2a were assessed by ELISA 24 hrs after the last challenge and expressed as optical density of diluted serum. Results were obtained from 3 individual experiments with 4 mice per group. ** P<0.01, # P<0.001, n.s. not significant. PBS/PBS/PE, sham-fed and sham-sensitized and peanut challenged; PBS/PE/PE, sham-fed and peanut sensitized and challenged; PE0.05/PE/PE, fed low doses of PE and PE sensitized and challenged; PE0.5/PE/PE, fed high doses of PE and PE sensitized and challenged.

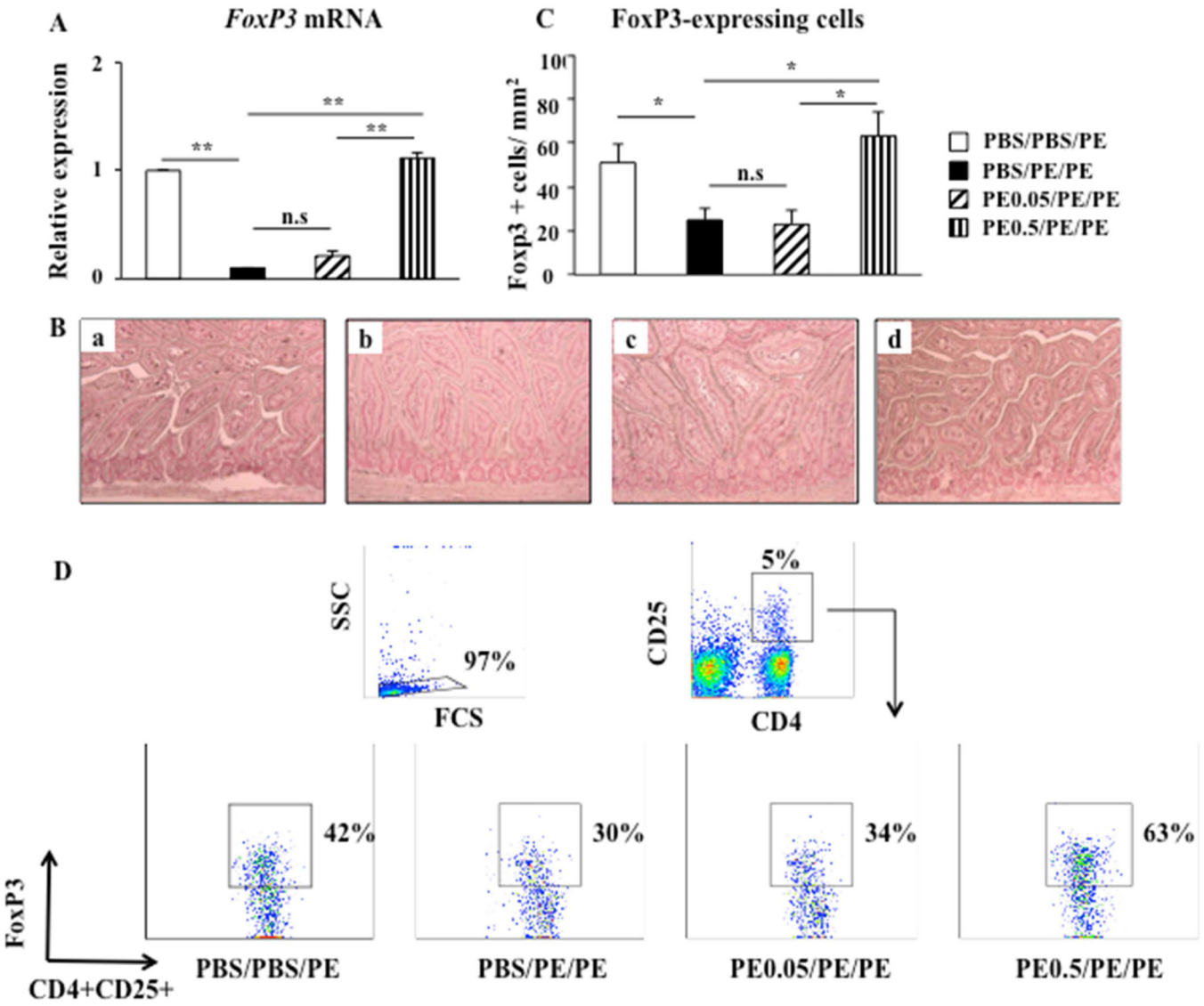


Figure 2.

Numbers of Tregs and FoxP3 expression in MLN CD4$^+$ T cells and jejunum. (A) *FoxP3* mRNA expression detected by quantitative RT-PCR in MLN CD4$^+$ T cells from PBS/PBS/PE, PBS/PE/PE, PE0.05/PE/PE, and PE0.5/PE/PE mice. (B) Mucosal FoxP3-expressing cells were identified following immunohistochemical staining with anti-FoxP3 antibody. Representative sections of (a) PBS/PBS/PE mice, (b) PBS/PE/PE mice, (c) PE0.05/PE/PE mice, (d) PE0.5/PE/PE mice. (C) Quantitation of mucosal FoxP3-expressing cells. (D) Representative flow cytometric analysis of the percentage of FoxP3-expressing cells among the CD4$^+$CD25$^+$ T cells from MLN. Results were obtained from 3 individual experiments with 4 mice per group. Magnification x200. **P<0.01, n.s. not significant.

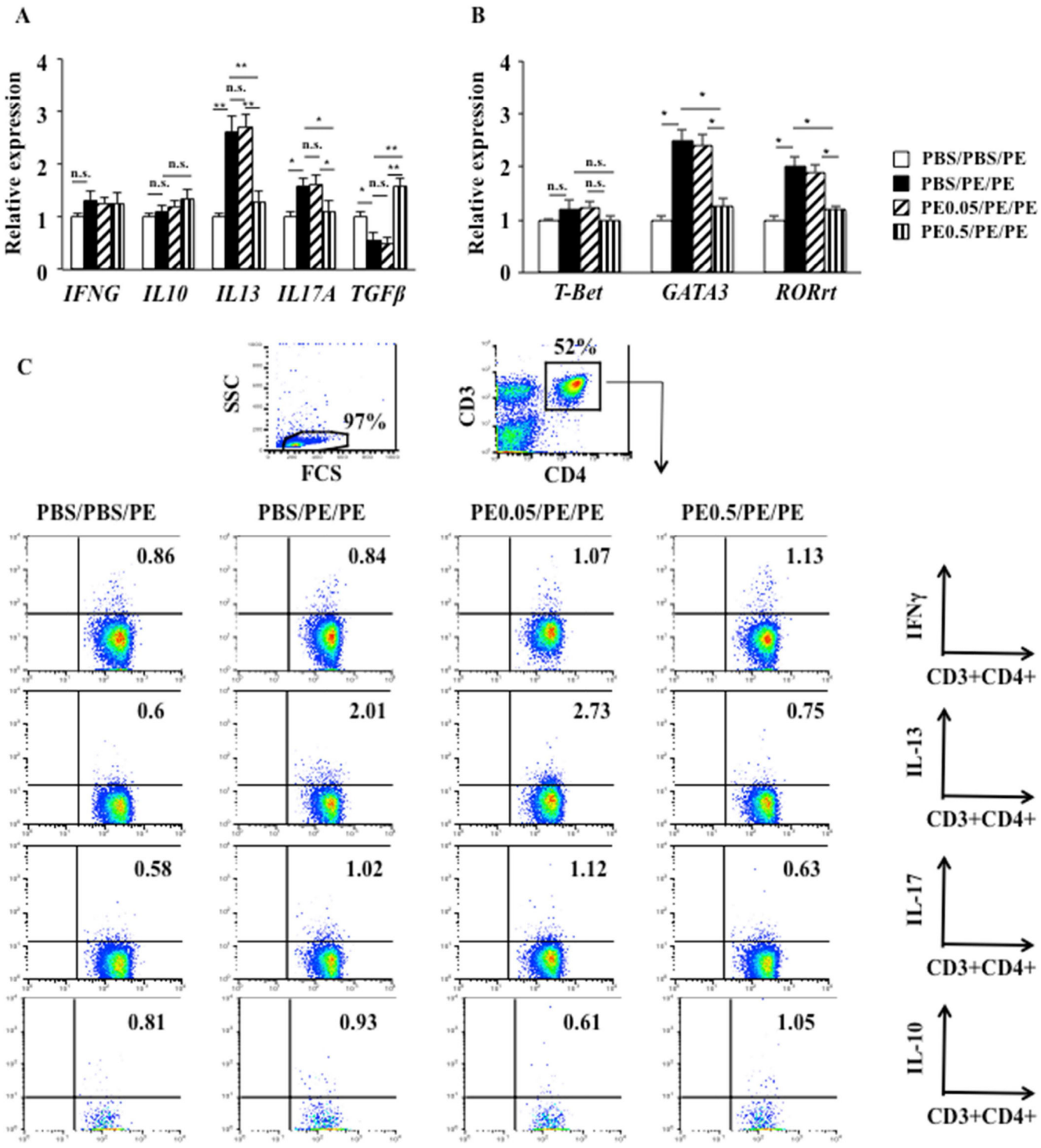


Figure 3.

Effects of tolerance induction on cytokine expression in MLN CD4$^+$ T cells and jejunum. (A) *IFNG, IL10, IL13, IL17A*, and *TGFβ* mRNA expression in jejunum. (B) *T-bet, GATA3*, and *RORγt* expression in jejunum. (C) Representative flow cytometric analysis of Th1, Th2,

and Th17 cytokine expression in $CD4^+$T cells from MLN. Results were from 3 independent experiments. *P<0.05; **P<0.01; n.s. not significant.

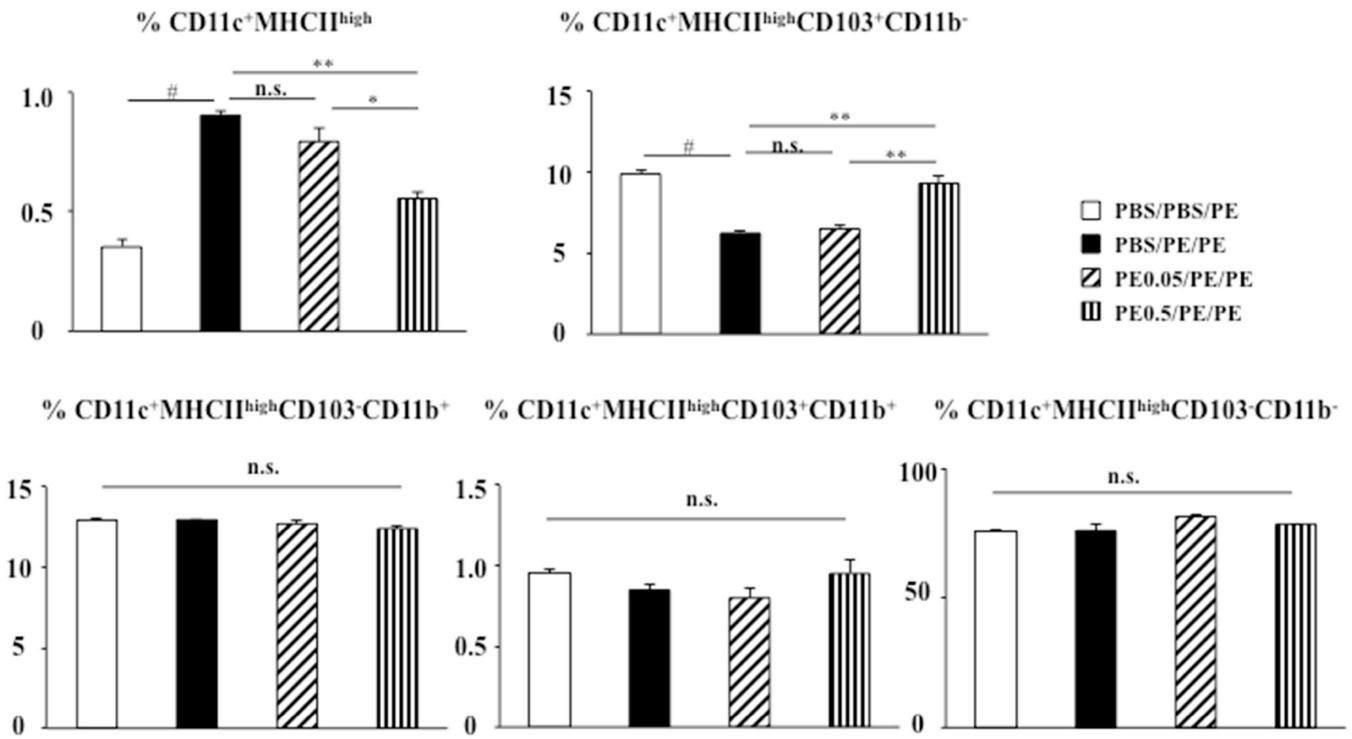


Figure 4.

DC subsets in mesenteric lymph node. Cells from MLN were stained with antibodies to identify DC subsets. The populations were gated on DAPI negative and B220 negative cells. The $CD11c^{+}MHCII^{high}$ population was further characterized on the basis of CD103 and CD11b expression. Results are from two independent experiments. *P<0.05; **P<0.01; #P<0.001; n.s. not significant.

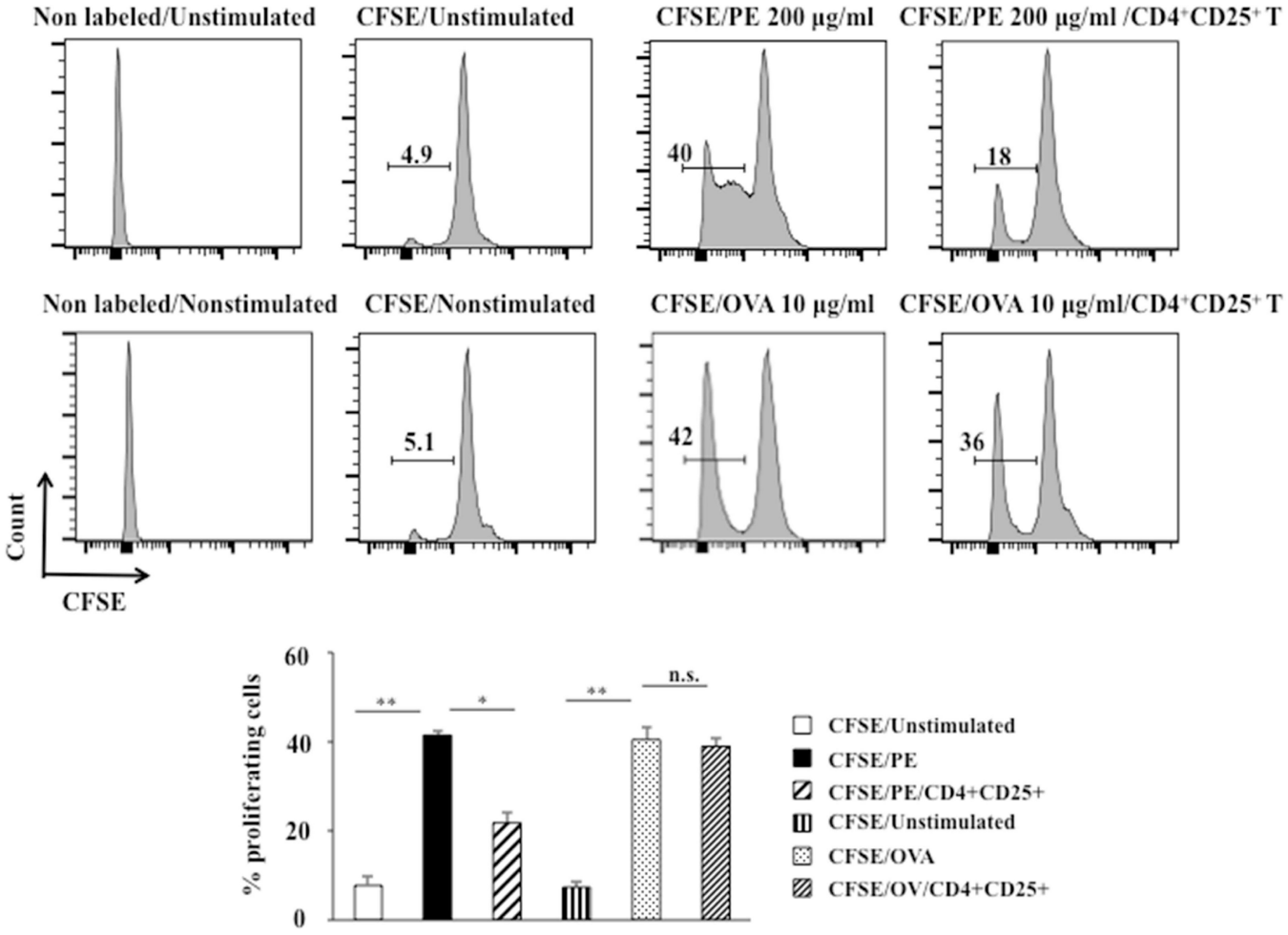
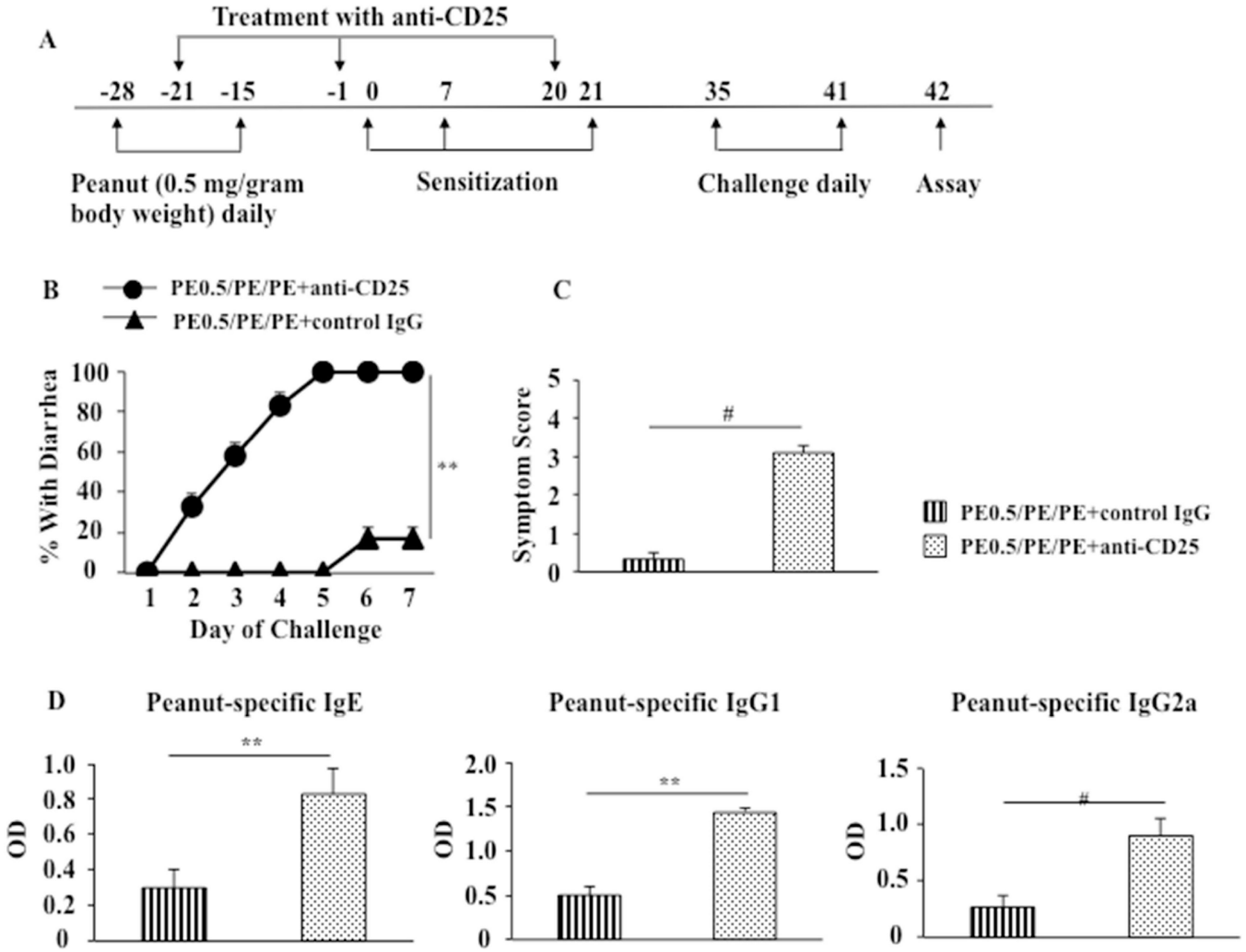


Figure 5.

Antigen-specific suppressive function of $CD4^+CD25^+$ cells. CFSE-labeled MNC from PE or OVA-immunized BALB/c mice were cocultured with $CD4^+CD25^+$ MLN cells from tolerant mice in the presence or absence of PE (200 μg/ml) or OVA (10 μg/ml) for 5 days. CFSE fluorescence intensities were monitored by flow cytometry. Results from two independent experiments carried out in triplicate are shown. *P<0.05, **P<0.01, n.s. not significant.



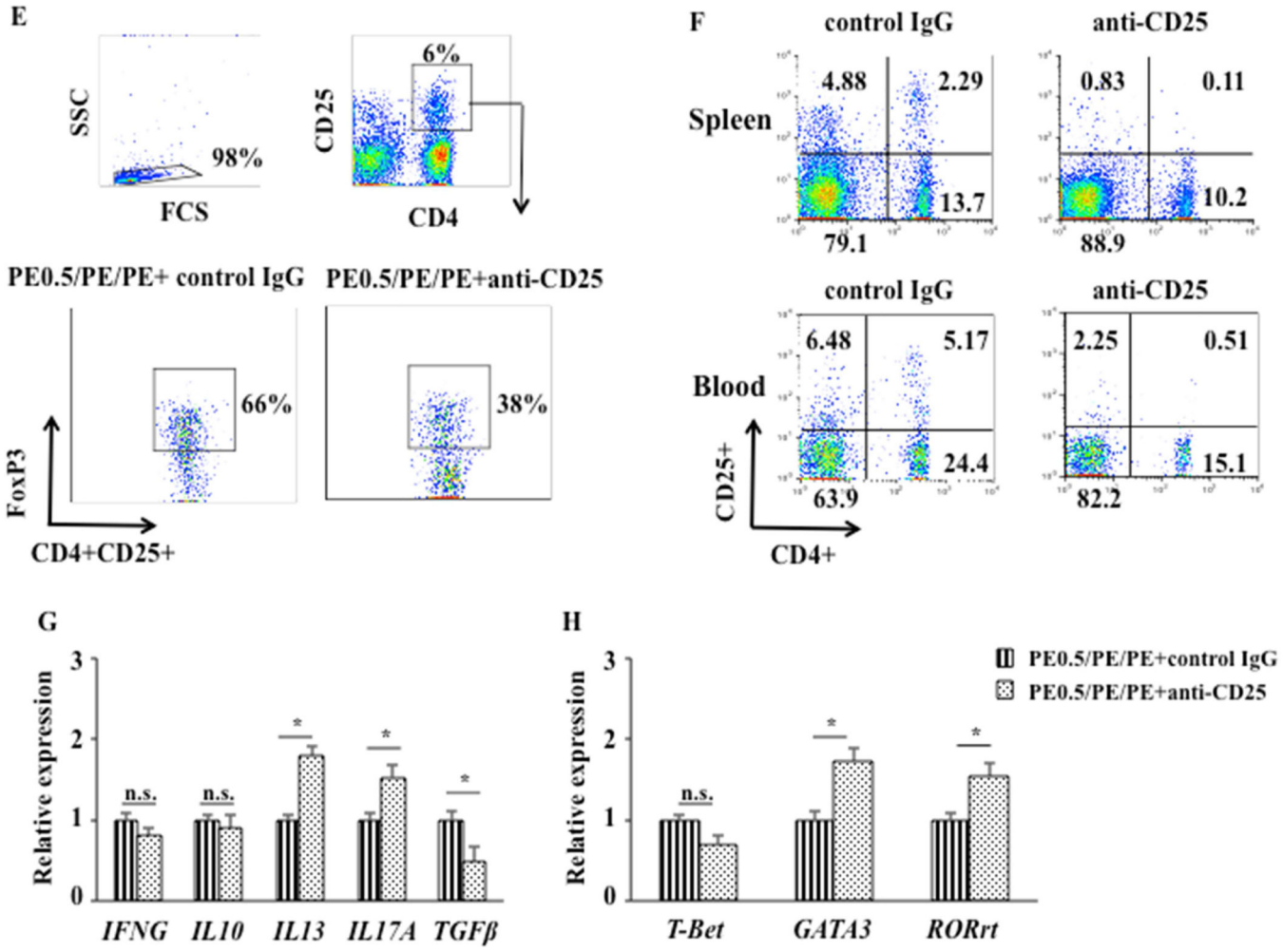


Figure 6.

Anti-CD25 administration attenuates oral tolerance. (A) Protocol for induction of tolerance to peanut protein and treatment with anti-CD25. Anti-CD25 was administered to pups on days -21, -1, and 20 (B) Kinetics of the development of diarrhea in peanut tolerant mice treated with anti-CD25 or control IgG. (C) Scores based on the severity of clinical signs were assessed 30 minutes after oral challenge. (D) Serum levels of peanut-specific IgE, IgG1, and IgG2a were assessed by ELISA 24 hrs after the last challenge and expressed as optical density of diluted serum. (E) Representative flow cytometric analysis of the percentage of FoxP3-expressing cells among the $CD4^+CD25^+$ T cells from MLN of tolerant mice treated with anti-CD25 or control IgG. (F) Representative flow cytometric analysis of the percentage of $CD4^+CD25^+$ T cells in spleen and peripheral blood from tolerant mice treated with anti-CD25 or control IgG. (G) *IFNG, IL10, IL13, IL17A*, and *TGFβ* mRNA expression in jejunum of tolerant mice treated with anti-CD25 or control IgG. (H) *T-bet, GATA3*, and *RORγt* expression in jejunum of tolerant mice treated with anti-CD25 or control IgG. Results were obtained from 2 individual experiments with 4 mice per group. *P<0.05, **P<0.01, #P<0.001, n.s. not significant.

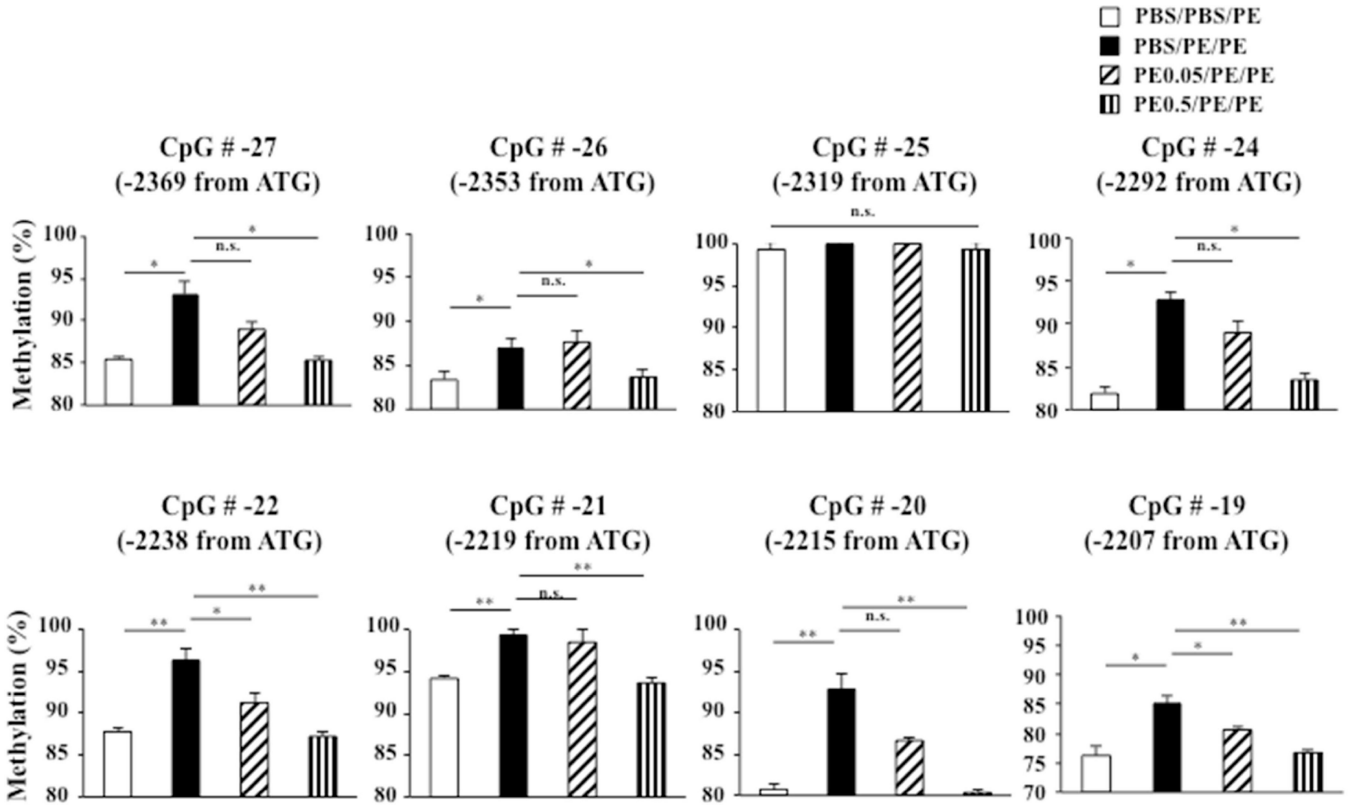


Figure 7.

FoxP3 DNA methylation in MLN CD4$^+$ T cells. FoxP3 methylation detected by bisulfite pyrosequencing was assayed in genomic DNA from MLN CD4$^+$ T cells; 9 CpG sites within the TSDR were examined. Results were obtained from 2 independent experiments. *P<0.05, **P<0.01, n.s. not significant.