

## Letter to the Editor

# Single allergen-induced oral tolerance inhibits airway inflammation in conjugated allergen immunized mice

To the Editor:

Studies in oral tolerance usually focus on the antigen-specific tolerogenic effect by using the same antigen as both the allergen and oral treatment; however, the major allergens of allergic

asthma are mixed and complicated. Very few reports have investigated the effect of oral tolerance to single allergen on the mixed allergen-induced asthma. Yasue et al<sup>1</sup> showed that oral administration of recombinant *Dermatophagoides farinae* group 2 allergen reduced recombinant *D. farinae* group 2 allergen-induced infiltration of neutrophils into airway but that effect could not be observed in crude mite extract-induced cellular infiltration. In the present study, we aimed to study whether a single allergen-

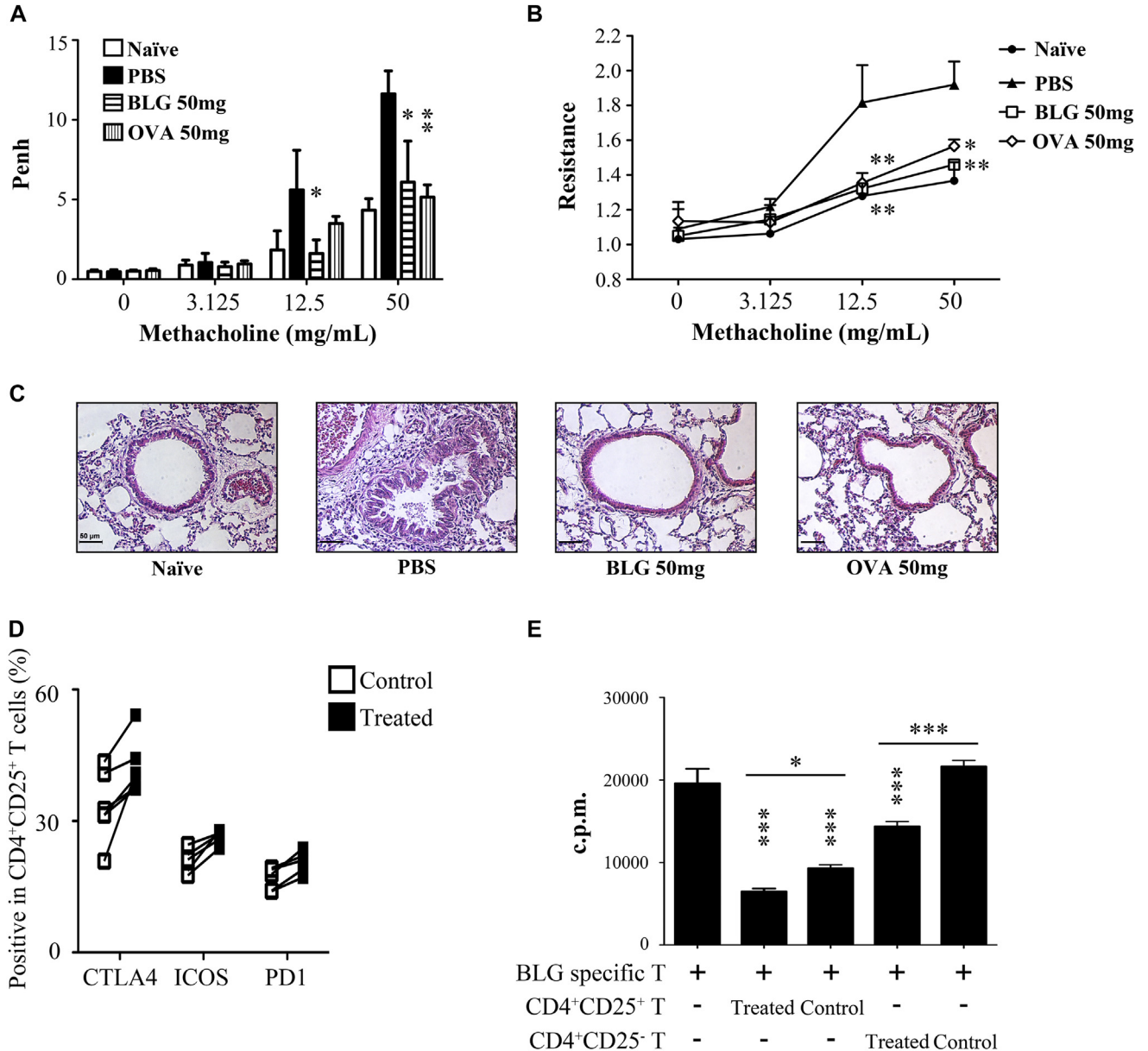


**FIG 1.** Preventive oral administration of antigen improved non-antigen-specific immune regulation and protected mice from related antigen-induced airway inflammation. **A-C**, BALB/c mice were sensitized with B-O, orally treated with PBS (PBS) or 50 mg of BLG (BLG 50 mg) or OVA (OVA 50 mg), and intranasally challenged with BLG. **A**, Airway hyperresponsiveness. **B**, Airway resistance. **C**, Lung sections. Scale bar, 50  $\mu$ m ( $n = 4-8$  per group). \* $P < .05$ ; \*\* $P < .01$ ; \*\*\* $P < .001$ , compared with the PBS group unless otherwise indicated. **D**, Expression in CD4<sup>+</sup>CD25<sup>+</sup> T cells purified from PBS-treated (control, white) and OVA-treated (treated, black) DO11.10 mice. **E**, Non-antigen-specific suppressive assay. \* $P < .05$ ; \*\* $P < .01$ ; \*\*\* $P < .001$ , compared with responder T-cell-only group unless otherwise indicated.

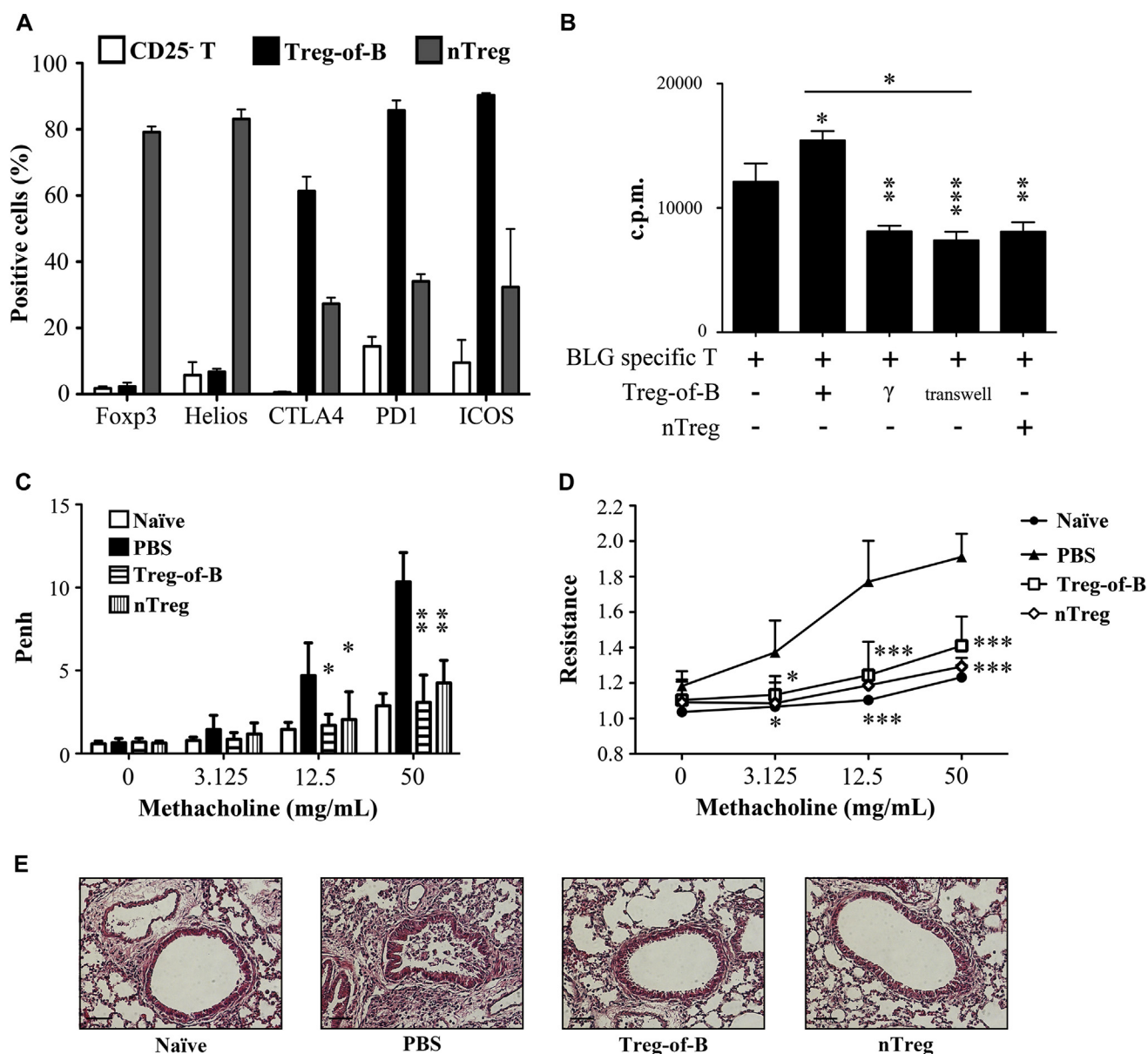


**FIG 2.** Characteristics of Treg-of-B cells correlated with orally treated CD4<sup>+</sup>CD25<sup>+</sup> T cells and protected mice from related antigen-induced airway inflammation. **A**, Expressions of FcγR3, Helios, CTLA4, PD1, and ICOS in CD4<sup>+</sup>CD25<sup>-</sup> T (white), Treg-of-B cells (black), and nTreg cells (CD4<sup>+</sup>CD25<sup>+</sup> T, gray). **B**, Non-antigen-specific suppressive assay. \**P* < .05; \*\**P* < .01; \*\*\**P* < .001, compared with the responder T-cell-only group unless otherwise indicated. γ, Cells were irradiated; *transwell*, with transwell insertion. **C–E**, Adoptive transfer of PBS or Treg-of-B (Treg-of-B) or CD4<sup>+</sup>CD25<sup>+</sup> (nTreg) cells into BALB/c mice before B-O sensitization and BLG intranasal challenge. **C**, Airway hyperresponsiveness. **D**, Airway resistance. **E**, Lung sections. Scale bar, 50 μm (n = 4–8 per group). \**P* < .05; \*\**P* < .01; \*\*\**P* < .001, compared with the PBS group unless otherwise indicated.

induced oral tolerance would regulate immune responses to other related allergens that were primed together with the orally treated allergen.

We investigated whether the oral administration of a single antigen would inhibit the other antigen-induced airway inflammation in conjugated antigen-sensitized mice. Preventive oral administration of ovalbumin (OVA) protected β-lactoglobulin (BLG)-conjugated-OVA (B-O)-sensitized mice from BLG-induced airway hyperresponsiveness (Fig 1, A and B, and see the Methods section in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

The non-antigen-specific tolerogenic effect was also observed in BLG-treated and OVA-challenged mice (see Fig E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)), which suggested a non-antigen-specific regulation of oral tolerance. The orally OVA-treated CD4<sup>+</sup>CD25<sup>+</sup> T cells had higher percentages of cells expressing cytotoxic T-lymphocyte-associated antigen 4 (CTLA4), inducible T-cell costimulator (ICOS), and programmed cell death 1 (PD1) and produced higher levels of IL-10, TGF-β, and IFN-γ than did PBS-treated control CD4<sup>+</sup>CD25<sup>+</sup> T cells

(Fig 1, D, and see Fig E2, A, in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). These characteristics improved their suppressive function in non-antigen-specific and antigen-specific manners (Fig 1, E, and see Fig E2, B). These results suggested that orally activated CD4<sup>+</sup>CD25<sup>+</sup> T cells exhibited regulation to other antigen-specific effector T cells via the concurrent presence of both antigens at the same position; thus, the non-antigen-specific regulation of oral tolerance could be observed in our conjugated antigen-sensitized animal model.

Our results suggested that oral treatment enhanced the suppressive function of CD4<sup>+</sup>CD25<sup>+</sup> T cells and there might be non-naturally occurring regulatory T (nTreg) cells in orally activated CD4<sup>+</sup>CD25<sup>+</sup> T cells. We further investigated whether splenic B cells, the major cell population in the spleen, had a role in oral tolerance. B-cell-deficient mice had a defect in oral tolerance and demonstrated decreased levels of IL-10 and TGF- $\beta$  in the spleen.<sup>2</sup> Studies showed that splenic B cells had a chance to present antigens derived from the oral route to CD4<sup>+</sup> T cells<sup>3</sup> and B cells had an ability to induce regulatory T cells *in vitro*.<sup>4-6</sup> In accordance with these, we found that adoptive transfer of OVA<sub>323-339</sub> peptide-pulsed B cells into naive DO11.10 mice decreased the proliferation of splenocytes to OVA<sub>323-339</sub> peptide *ex vivo* (data not shown). OVA<sub>323-339</sub> peptide-pulsed splenic B cells converted CD4<sup>+</sup>CD25<sup>-</sup> T cells into CD4<sup>+</sup>CD25<sup>+</sup> T cells (referred to as Treg-of-B cells) *in vitro* with expressions of ICOS and PD1 (Fig 2, A, and see Fig E3, A, in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)), which corresponded to orally treated CD4<sup>+</sup>CD25<sup>+</sup> T cells (Fig 1, D). Treg-of-B cells did not express forkhead box P3 (Foxp3) or Helios, suggesting that they were not a subpopulation of nTreg cells. Antigen-activated Treg-of-B cells produced higher levels of IL-10 and TGF- $\beta$ , which might be involved in their suppressive functions (see Fig E3, B and C). Treg-of-B cells exhibited a non-antigen-specific suppressive function via a contact-independent manner (Fig 2, B) and an antigen-specific suppressive function via a partially contact-dependent manner (see Fig E3, C). Adoptive transfer of Treg-of-B cells protected conjugated antigen-sensitized mice from the other antigen-induced airway inflammation (Fig 2, C and D) and cellular infiltration (Fig 2, E). Our results suggested that several characteristics of Treg-of-B cells correlated with orally treated CD4<sup>+</sup>CD25<sup>+</sup> T cells, including higher expressions of ICOS, PD1, IL-10, and TGF- $\beta$  as well as the non-antigen-specific suppressive function.

Our results suggested that preventive oral administration of a single allergen had a beneficial effect on immune responses to other related allergens that were primed together with the orally treated allergen. Karlsson et al<sup>7</sup> demonstrated that oral administration of 9% OVA in drinking water before sensitization inhibited both OVA- and human serum albumin (HSA)-induced anaphylaxis. This non-antigen-specific regulatory effect could be observed in the mice sensitized with OVA and HSA in the same leg but not in the mice sensitized with OVA and HSA in different legs.<sup>7</sup> Suzuki et al<sup>8</sup> demonstrated that prophylactic oral administration of approximately 50 mg of a fragment of

*Dermatophagoides pteronyssinus* group 1 allergen over 7 days decreased allergic asthma induced by the same allergen but the animal administered with OVA did not. These results suggested that non-antigen-specific regulatory effect of oral tolerance could be detected in the condition that active regulatory T cells and effector T cells concurrent in the same position; thus, non-antigen-specific regulation of oral tolerance could be more clearly detected in conjugated antigen-sensitized mice.

We found that characteristics of Treg-of-B cells correlated with orally treated CD4<sup>+</sup>CD25<sup>+</sup> T cells, suggesting that splenic B cells might present antigen for primary CD4<sup>+</sup> T-cell responses in the establishment of oral tolerance. Our results implied that preventive oral administration of a single antigen might be useful to inhibit whole allergen-induced allergic asthma. The present study was one of few reports that have investigated the non-antigen-specific effect of oral tolerance and might further our understanding of applications of oral tolerance induced by single allergen in allergic asthma.

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## METHODS

### Animals

Female BALB/c mice were purchased from the National Laboratory Animal Center. The transgenic T-cell receptor of DO11.10 mice recognizes the OVA<sub>323-339</sub> peptide presented by H-2<sup>d</sup>. All mice used were aged between 6 and 12 weeks and maintained in specific pathogen-free conditions at Laboratory Animal Center of College of Medicine at National Taiwan University. The Animal Research Committee of the College of Medicine at National Taiwan University approved all animal experiments.

### Generation of B-O antigen

BLG (L3908; Sigma-Aldrich, St Louis, Mo) and OVA (A5503; Sigma-Aldrich) were conjugated by linker (MAL-PEG<sub>8</sub>-NHS, 22104; Thermo Scientific, Waltham, Mass) according to the manufacturer's instructions. Briefly, BLG was conjugated with linker, and then incubated with reduced OVA at a molar ratio of 1:1. To remove unbound OVA or BLG, conjugations were applied to a centrifugal filter (Amicon Ultra-15 unit with ultracel-50 membrane; Millipore, Billerica, Mass) and size exclusion (TOYOPEARL HW-50F, TOSOH, Tokyo, Japan) chromatography (BioLogic LP System; Bio-Rad, Hercules, Calif). Western blot was performed to confirm conjugation without free BLG or OVA.

### Allergic asthma and adoptive transfer

For allergic asthma induction, BALB/c mice were intraperitoneally injected with 10 µg of B-O in aluminum adjuvant (77161; Thermo Scientific) 4 times and were intranasally challenged with 100 µg of BLG or OVA. For adoptive transfer,  $5 \times 10^6$  Treg-of-B cells or  $1 \times 10^6$  nTreg cells were injected into the tail vein before sensitization and challenge.

### Measurement of airway hyperresponsiveness and airway resistance

The lung function of mice was determined by measuring airway responses to different doses of acetyl-β-methylcholine chloride (methacholine; A2251; Sigma-Aldrich) in a barometric whole-body plethysmographic chamber (FinePointe system; Buxco Electronics, Wilmington, NC). After inhalation of nebulized methacholine for 3 minutes, the respiratory pressure curves were recorded for the next 3 minutes. The averages of methacholine-induced airway obstruction were presented as plethysmography and enhanced pause values;  $P_{enh} = \text{pause} \times (\text{peak expiratory box flow/peak inspiratory box flow})$ .

The airway resistance of mice was determined by measuring responses to aerosolized PBS containing increasing concentrations of methacholine (3.125–50 mg/mL) in an invasive body plethysmography. Mice were anesthetized and tracheally cannulated and ventilated (150 breaths/min; tidal volume, 0.3 mL). Airway resistance was calculated by using a software program (Model PNM-PCT100W, LDS PONEMAH Physiology Platform; LDS GOULD, Valley View, Ohio). The resistance of the orotracheal tube was subtracted from all airway resistance measurements.

### Analysis of orally treated CD4<sup>+</sup>CD25<sup>+</sup> T cells

DO11.10 mice were sensitized with OVA at day 1 and orally treated with 50 mg of OVA (treated) or PBS (control) for 5 consecutive days. Spleen, mesenteric lymph node, and Peyer's patches were harvested at day 6 and filtered directly through a 70-µm strainer (BD, Franklin Lakes, NJ). CD4<sup>+</sup>CD25<sup>+</sup> and

CD4<sup>+</sup>CD25<sup>−</sup> cells were purified by immunomagnetic selection using magnetic beads–conjugated antibodies (mouse CD4 T-lymphocyte enrichment set, 558131; anti-PE magnetic particles, 557899; BD). Positive or negative selections were performed to purify each cell population according to the manufacturer's instructions. The purity of the cells was confirmed by flow cytometry and was at least 90%.

### Treg-of-B cells

Splenic B220<sup>+</sup> cells from naive BALB/c mice were purified and pulsed with OVA<sub>323-339</sub> peptide (5 µg/mL). After 1 day, splenic CD4<sup>+</sup>CD25<sup>−</sup> cells from naive DO11.10 mice were purified and cultured with B cells at a ratio of 1:1 for 3 days. Treg-of-B cells were collected by depleting B220<sup>+</sup> cells.

### Enriched BLG-specific T cells

The splenocytes were harvested from BLG-sensitized mice, and cultured with BLG (100 µg/mL) for 3 days. The CD4<sup>+</sup> T cells were purified by magnetic-beads–conjugated antibodies (anti-mouse CD4 magnetic particles, 551539; BD), and rested in recombinant IL-2 (50 U/mL) for another 3 days before suppressive assay.

### <sup>3</sup>H incorporation assay

For the antigen-specific suppressive assay,  $1 \times 10^5$  DO11.10 splenic CD4<sup>+</sup>CD25<sup>−</sup> T cells as responder cells were stimulated with γ-irradiated (3000 rads) and OVA<sub>323-339</sub> peptide-pulsed cells. For the non-antigen-specific suppressive assay, enriched BLG-specific CD4<sup>+</sup> T cells were stimulated with γ-irradiated, BLG-treated, and OVA<sub>323-339</sub> peptide-pulsed cells. The suppressor cells were seeded with responder cells at a ratio of 1:1. [<sup>3</sup>H]-thymidine (1 µCi/well, PerkinElmer, Boston, Mass) was added after 3 days and the cells were harvested 16 to 18 hours later. The results were presented in counts per minute.

### Cytokine analysis

The purified cells were seeded at  $1 \times 10^6$  cells/mL and were stimulated with irradiated and OVA<sub>323-339</sub> peptide-pulsed cells for 48 hours. Cytokine levels in the culture media were measured by ELISA kit (DuoSet ELISA Development kit, R&D, Minneapolis, Minn) according to the manufacturer's instructions.

### Flow cytometry

Flow cytometry was performed according to standard procedures. Data acquisition was performed on a FACSCalibur (BD), and data were analyzed using the CellQuest Pro software (BD). The following fluorescent-labeled anti-mouse mAbs (and clone names) were obtained from eBioscience (San Diego, Calif): CD4 (GK1.5), CD25 (PC61.5), ICOS (C398.4A), PD1 (J43), CTLA4 (UC10-4B9).

### Statistical analysis

Statistical analysis was performed with the Mann-Whitney test or 1-way ANOVA, where appropriate, by using GraphPad Prism 5 Software. A *P* value of less than .05 was considered statistically significant. All data were expressed as mean ± SD. Data were representative of at least 3 independent experiments except invasive airway hyperresponsiveness experiments, which were performed once for each experimental protocol (*n* = 4–8 per group).

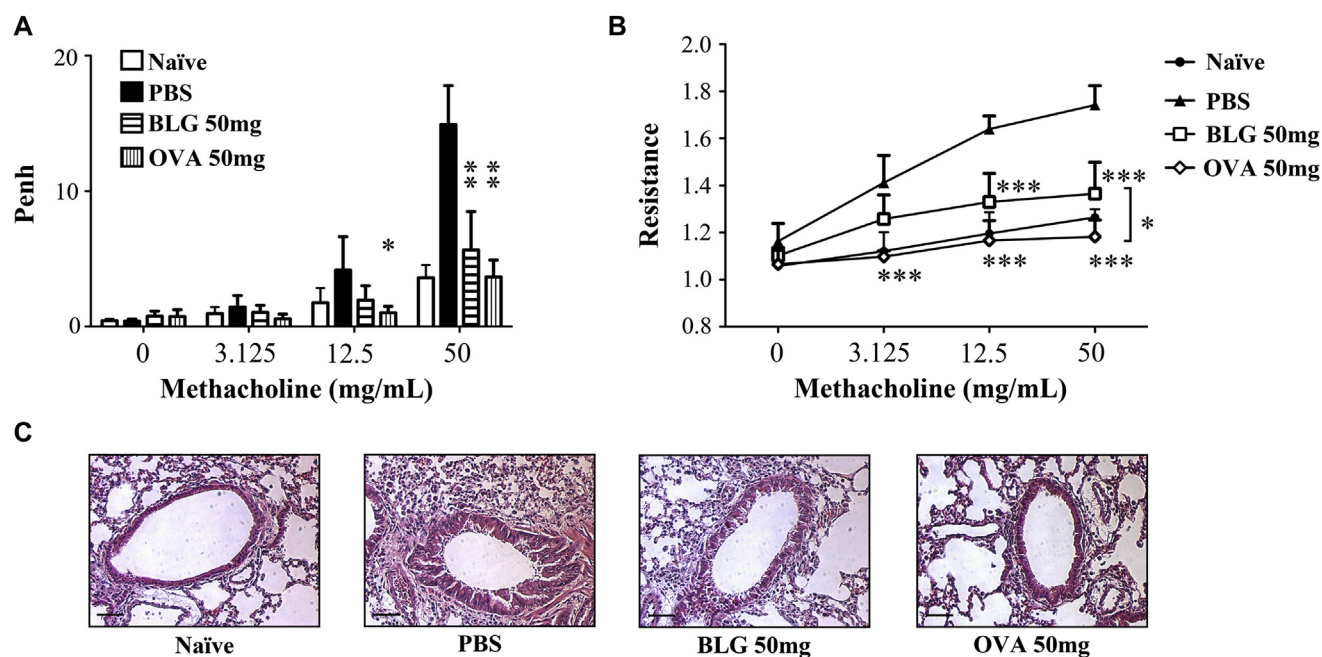


**FIG E1.** Inhibition of OVA-induced airway inflammation by prophylactic oral administration with BLG in B-O-sensitized mice. BALB/c mice were sensitized with B-O, orally treated with PBS (PBS) or 50 mg of BLG (BLG 50 mg) or OVA (OVA 50 mg), and intranasally challenged with BLG. **A**, Airway hyperresponsiveness. **B**, Airway resistance. **C**, Lung sections. Scale bar, 50  $\mu$ m (n = 4-6 per group). \* $P$  < .05; \*\* $P$  < .01; \*\*\* $P$  < .001, compared with the PBS group unless otherwise indicated.

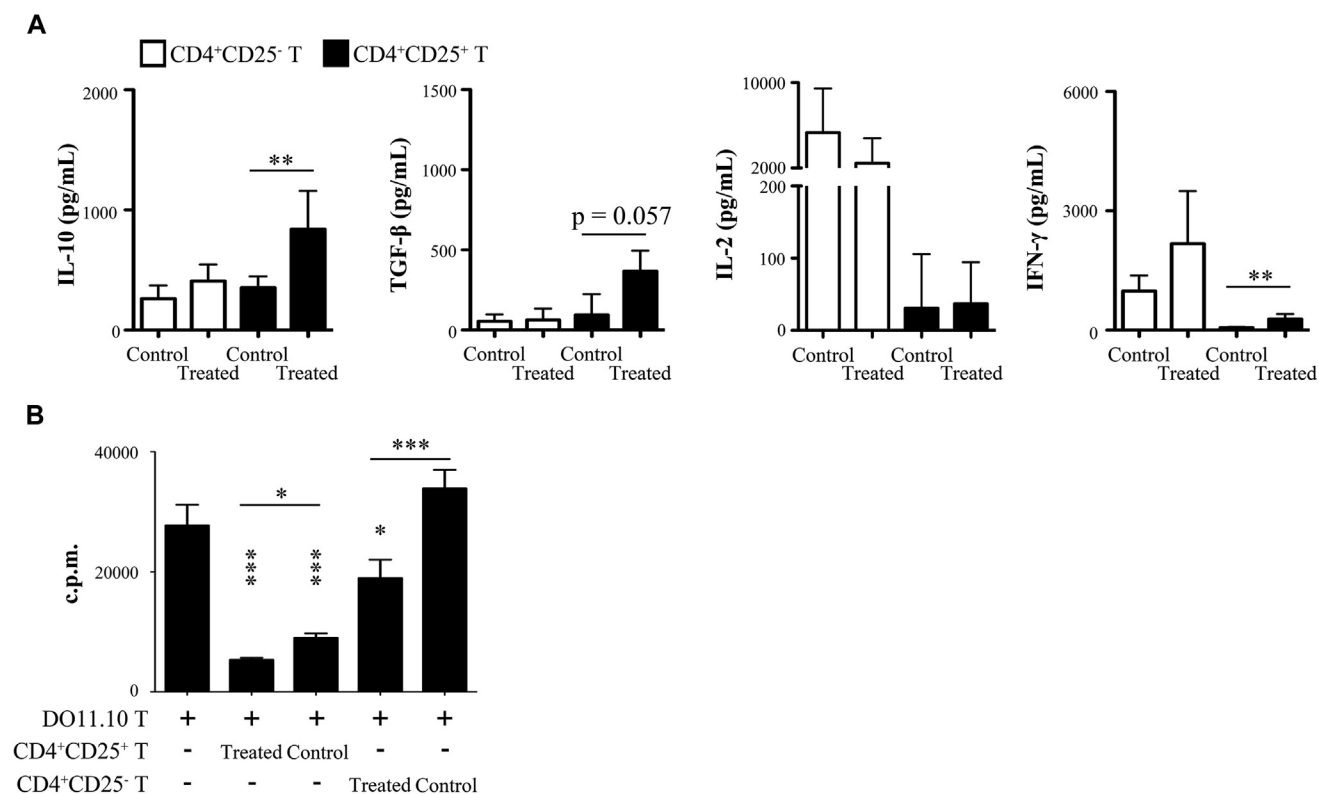


**FIG E2.** Oral administration of antigens improved the suppressive function of CD4<sup>+</sup>CD25<sup>+</sup> T cells. CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> T cells from orally treated PBS (control) or 50 mg of OVA (treated) DO11.10 mice were purified. **A**, Cytokine levels of activated CD4<sup>+</sup>CD25<sup>-</sup> (white) and CD4<sup>+</sup>CD25<sup>+</sup> T (black) T cells. **B**, Antigen-specific suppressive assay. \* $P < .05$ ; \*\* $P < .01$ ; \*\*\* $P < .001$ , compared with responder T-cell-only group unless otherwise indicated.

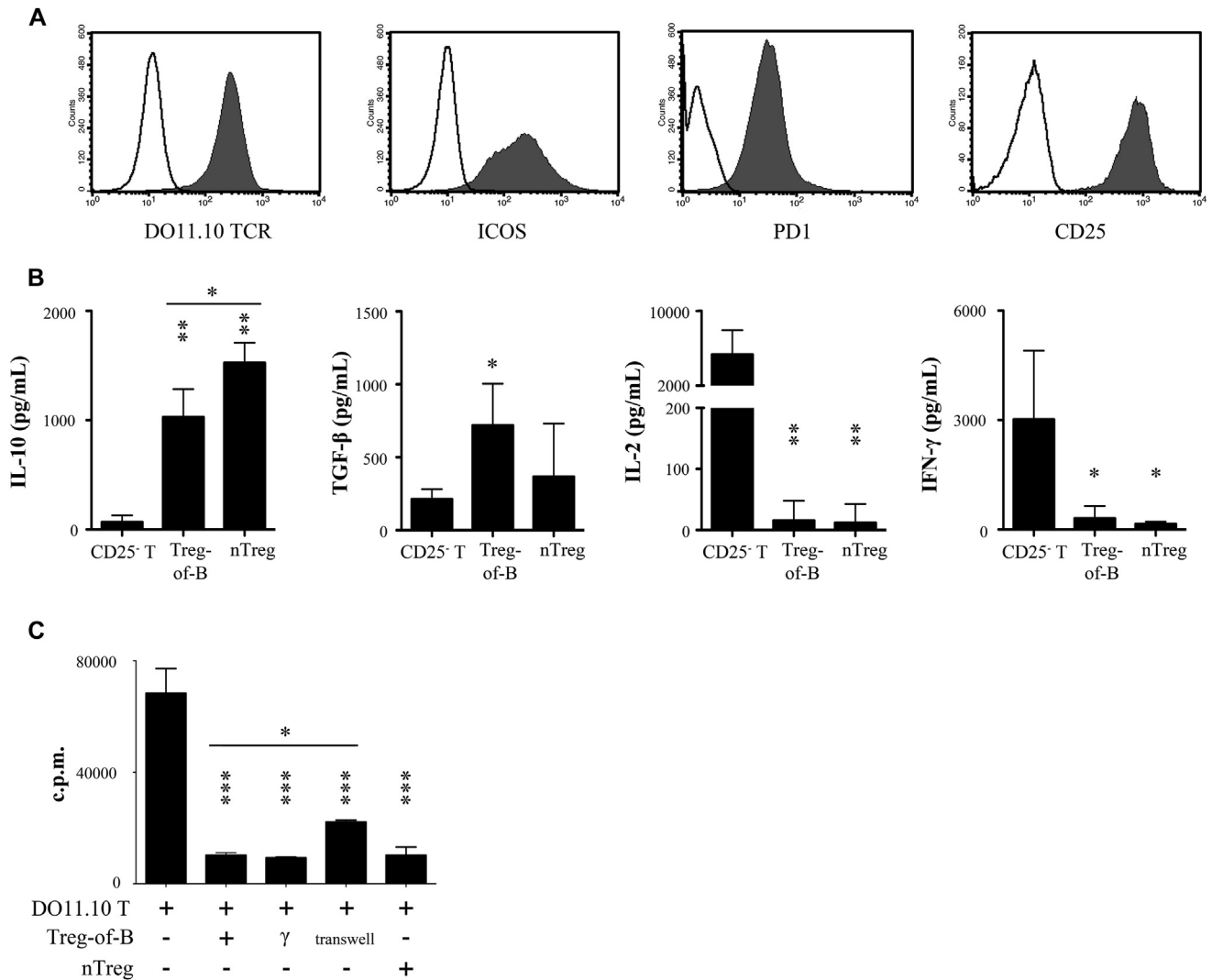


**FIG E3.** B cells had the ability to convert CD4<sup>+</sup>CD25<sup>-</sup> T cells into CD4<sup>+</sup>CD25<sup>+</sup> T cells with regulatory functions. **A**, Expression of Treg-of-B cells (filled) and isotype control (open). **B**, Cytokine levels of antigen-specific activated CD4<sup>+</sup>CD25<sup>-</sup> (CD25<sup>-</sup> T), Treg-of-B, and CD4<sup>+</sup>CD25<sup>+</sup> T (nTreg) cells. **C**, Antigen-specific suppressive assay. \* $P < .05$ ; \*\* $P < .01$ ; \*\*\* $P < .001$ , compared with the responder T-cell-only group unless otherwise indicated.  $\gamma$ , Cells were irradiated; *transwell*, with transwell insertion.