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ORIGINAL ARTICLE

Intranasal ovalbumin immunotherapy with mycobacterial adjuvant promotes regulatory T cell accumulation in lung tissues

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ABSTRACT

Allergen-specific immunotherapy to induce T regulatory cells in the periphery has been used to treat allergic diseases. *Mycobacteria* can be used as an adjuvant for inducing T regulatory cells. However, it is unclear whether intranasal immunotherapy in combination with *Mycobacteria* adjuvant induces regulatory T cell differentiation and attenuates allergic responses *in vivo*. To investigate the role of intranasal ovalbumin (OVA) treatment alone and in combination with *Mycobacteria vaccae*, proportions of FoxP3^{+} regulatory T cells and anti-inflammatory responses were evaluated in a murine model of asthma that was established in three groups of bicistronic Foxp3^{EGFP} reporter BALB/c mice. Before establishment of the asthma model, two groups of mice received intranasal OVA immunotherapy and one also received simultaneous s.c. *M. vaccae*. Expression of CD4^{+}CD25^{+}Foxp3^{+EGFP+} T cells in the lung and spleen was analyzed by flow cytometry and the cytokine profiles of allergen-stimulated lung and spleen lymphocytes assessed. The intranasal OVA immunotherapy group showed greater expression of CD4^{+}CD25^{+}Foxp3^{+EGFP+} T cells in the spleen whereas in the group that also received *M. vaccae* such greater expression was demonstrated in the lung. Additionally, the proportion of IL-10 and IFN-γ-secreting splenocytes was greater in the intranasal OVA + *M. vaccae* group. CD25 neutralization decreased CD4^{+}Foxp3^{+} cells more than other groups. In parallel with this finding, production of IL-10 and IFN-γ was down-regulated. Mucosal administration of OVA antigen results in a greater proportion of CD4^{+}Foxp3^{+} T cells in the spleen. IL-10 and IFN-γ induced by intranasal OVA immunotherapy and *M. vaccae* administration is down-regulated after CD25 neutralization.

Key words allergen-specific immunotherapy, asthma model, BALB/c, Foxp3, Treg cells.

CD4^{+}CD25^{+}Foxp3^{+} Treg cells have immunosuppressive/immunomodulatory capacity and can reportedly prevent generation of immune response to self-antigens and innocuous environmental antigens, including allergens in the periphery (1). Moreover, Foxp3 expression has been shown to be significantly negatively correlated with IgE, eosinophilia and Foxp3^{+}/CD4^{+} ratio in patients with asthma and atopic dermatitis (2).

Allergen-specific mucosal immunotherapy has been used in recent decades in treatment of respiratory allergic diseases. Induction of a tolerant state in peripheral T cells is an essential step in allergen specific immunotherapy (3). Peripheral T-cell tolerance is characterized mainly by generation of allergen-specific

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List of Abbreviations: EGFP, enhanced green fluorescent protein; OVA, ovalbumin; TGF-β, Transforming growth factor-β; Treg, T regulatory cells.

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Treg cells and suppresses proliferative and cytokine responses against the major allergen (4).

Currently, immunotherapy based on both immune deviation and immune suppression, including enhancement of regulatory T cells, offers attractive strategies for treating asthma. $CD4^{+}CD25^{high}$T cells are essential for establishing tolerance to allergen-specific activated $CD4^{+}$T cells (5).

Several studies have suggested that mycobacteria can be used as an adjuvant for inducing Tregs. Treatment of mice with mycobacterium-induced allergen-specific Tregs induces IL-10 and TGF-β, which protect against airway inflammation (2). Our previous research supported the role of *M. vaccae* administration in preventing development of asthma during both pregnancy and the neonatal period in mice (6). Another recent study has shown that probiotics also have immunomodulatory properties as adjuvants with skewing of $CD4^{+}$T cells towards a Th1/possibly Treg or Th1 profile (7).

The current study aimed to explore the distribution of Treg cells in the context of mucosal immunotherapy with and without the use of an adjuvant by using $Foxp3^{+EGFP+}$ bicistronic mice in an experimental asthma model and investigating the involvement of those cells by assessing CD25 depletion.

MATERIALS AND METHOD

Mice

Five to 6-week-old healthy BALB/c mice that carry a bicistronic $Foxp3^{+EGFP+}$ gene that directs the couple expression of both Foxp3 and EGFP were kindly provided by Professor Talal Chatila (University of California, Los Angeles, CA, USA). All animals were housed according to the National Institutes of Health Guidelines. Experimental protocols were approved by our institution's ethics committee and complied with the guidelines of the Animal Research Committee of Marmara Medical University (Istanbul, Turkey).

Study design

An asthma model was established in three groups each of five to seven mice by administering intraperitoneal OVA (10 μg) for 2 days followed by seven nebulizations of OVA (1%) (Fig. 1). Group I $Foxp3^{+EGFP+}$ bicistronic BALB/c mice received 30 μL PBS containing 100 μg OVA (Type V: Sigma, St Louis, MO, USA) intranasally on six consecutive days (Days 1–6) (IN-OVA group). Group II $Foxp3^{+EGFP+}$ bicistronic BALB/c mice received s.c. heat-killed 1×10^{7} CFU *M. vaccae* three times on Days 1, 3 and 5 in addition to the same intranasal immunotherapy as Group I (IN-OVA + *M. vaccae* group). Group III served as a control asthma model and Group IV as healthy controls.

Expression of $CD4^{+}Foxp3^{+EGFP+}$ T cells in spleen and lung lymphocytes was analyzed by flow cytometry. Splenocytes and lung lymphocytes from all groups were cultured and cytokine production determined by ELISA. IL-10 and IFN-γ secreting T cells were quantitated by magnetic separation with Automacs (Miltenyi Biotec AG, Bergisch Gladbach, Germany)

$CD4^{+}$ $CD25^{+}$ T cells were also depleted in the IN-OVA + *M. vaccae* group.

M. vaccae preparation and treatment

Mycobacterium vaccae (ATCC 29678 strain) was cultured in Lowenstein–Jensen medium (Remel, Lenexa, KS, USA). The vials were autoclaved at 120°C for 25 min. Aliquots of 1×10^{7} CFU/mL in medium for s.c. administration were prepared and frozen at −20°C. Mice in group II (IN-OVA + *M. vacccae*) received 1×10^{7} CFU *M. vaccae* s.c. on the first, third and fifth days.

Cell culture

Spleens and lungs were removed from all groups of mice and cut into small pieces, after which single-cell suspensions and mononuclear cells were purified by means of Histopaque gradient centrifugation (Sigma, St. Lois MO, USA). Two to three spleens were pooled in each group. The cells were washed, counted and suspended in RPMI 1640 tissue culture medium (Sigma) containing heat-inactivated 10% FCS, 100 mg/mL penicillin, 100 mg/mL streptomycin, 5 mmol/L L-glutamine and 50 mmol/L 2-mercaptoethanol (all from Sigma). The splenocytes and lung lymphocytes were then stimulated with OVA (40 μg/mL), phytohemagglutinin (1 μg/mL; Sigma) and *M. vaccae* (2×10^{7} CFU/mL). Supernatants were harvested after 24 hr (for IL-10 and IFN-γ) or 48 hr (for IL-5). Amounts of cytokines in culture supernatants were determined with a commercial ELISA kit (Endogen, Rockford, IL, USA).

Isolation of IFN-γ-secreting and IL-10-secreting T cells

After purification of splenocytes, cells were stimulated with OVA for 16 hr, after which they were washed and labeled for 10 min with IFN-γ or IL-10 catch reagent in ice cold medium (Miltenyi Biotec AG). The cells were then suspended and allowed to secrete IFN-γ or IL-10 in closed tubes for 45 min at 37°C under slow continuous rotation. After capturing secreted cytokines at their surfaces, the cells were centrifuged at 300 g for 5 min at

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4°C and resuspended in cold PBS. They were then stained with PE-conjugated anti-IFN-γ and PE-conjugated anti-IL-10 for 20 min at 4°C. IFN-γ- and IL-10-secreting T cells were analyzed by flow cytometry before enrichment. The enrichment procedure was as follows: the cells were washed and resuspended in 80 μL buffer and magnetically labelled for 15 min at 4°C with 20 μL microbead-labeled anti-PE mAb and then enriched by positive and negative selection. The purity of IFN-γ+ and IL-10+ T cells was more than 82% in all experiments.

Purification of T cells and anti-CD3 stimulation

Following isolation of splenocytes and lung lymphocytes, pure T cells were isolated using a mouse pan T cell isolation kit (Miltenyi Biotec AG). Briefly, anti-CD14, anti-CD16, anti-CD19, anti-CD56, anti-CD36, anti-CD123, anti-CD235a were added to splenocytes to deplete B cells, NK cells, dendritic cells, monocytes, granulocytes and erythroid cells. Control Abs comprised PE- or FITC-conjugated mouse IgG1 and mouse IgG2 (BD PharMingen, San Jose, CA, USA). Fluorescence analysis was performed on an EPICS XL (Becman Coulter, Fullerton, CA, USA) with argon laser (488 nm). Isolated T cells were stimulated in T-cell activation 96-well assay plates (Anti-mouse CD3; BD Bioscience, San Jose, CA, USA). Three days after stimulation, the supernatants were collected and IFN-γ cytokines measured by ELISA.

Determination of $CD4^+$ $CD25^+$ $Foxp3^{+EGFP+}$ T cells

$CD4^+CD25^+$ $Foxp3^{+EGFP+}$ T cells among splenocytes and lung lymphocytes were analyzed via flow cytometry. Isolated cells were stained with anti-CD4 PE and anti-CD25 APC antibodies (E-Bioscience, San Diego, USA), after which flow cytometric analysis was performed.

Depletion of $CD4^+CD25^+$ Treg cell

In a separate experiment, $CD4^+CD25^+$ Treg cells were depleted by using anti mouse CD25 mAb PC 61 *in vivo* (E-Bioscience) as follows. Mice were treated i.p. with 170 μg/mL PC61 or rat IgG1 isotype control one day before the administration of IN-OVA + *M. vaccae*. After the mice had been humanely killed, expression of spleen $CD4^+CD25^+Foxp3^+$ T cells was analyzed by flow cytometry. OVA-specific serum IgE and IgG1 were quantitated by ELISA. Finally, amounts of IL-10 and IFN-γ of *M. vaccae* and phytohemagglutinin-stimulated splenocytes and lung lymphocytes were measured as described earlier.

Statistical analysis

SPSS 10.1 (SPSS, Chicago, IL, USA) was used for statistical analyses. The Kruskal–Wallis test was used for comparisons between groups. When differences were statistically significant, the Mann–Whitney *U*-test was used for between-group comparisons. $P < 0.05$ was considered to denote statistical significance.

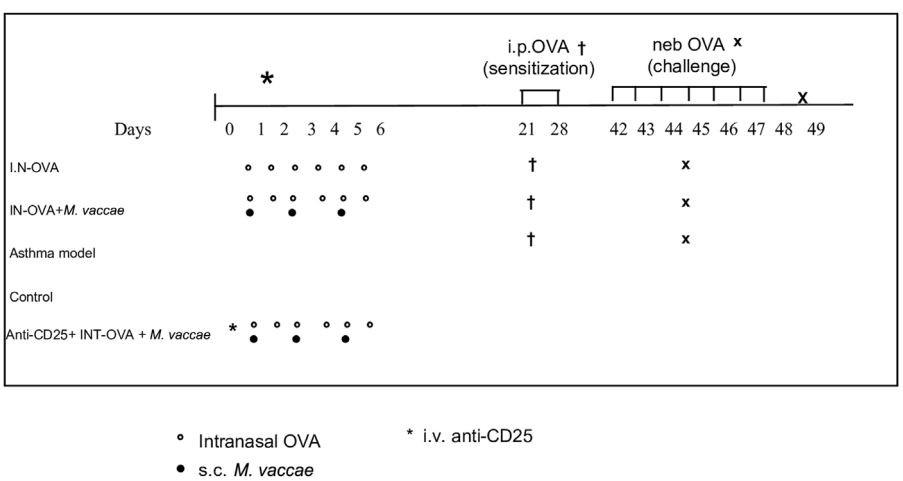

Fig. 1. Study design. The IN-OVA group received 100 μg OVA intranasally on Days 1–6 (once/day). IN-OVA + *M. vaccae* group received *M. vaccae* (1×10^7 CFU/100 μL) s.c. on Days 1, 3 and 5 and 100 μg OVA i.n. on Days 1-6. An asthma model was established by two intraperitoneal OVA injections and seven OVA nebulizations. CD25 neutralization was carried out on the day before intranasal treatment. The control group did not receive any treatment.

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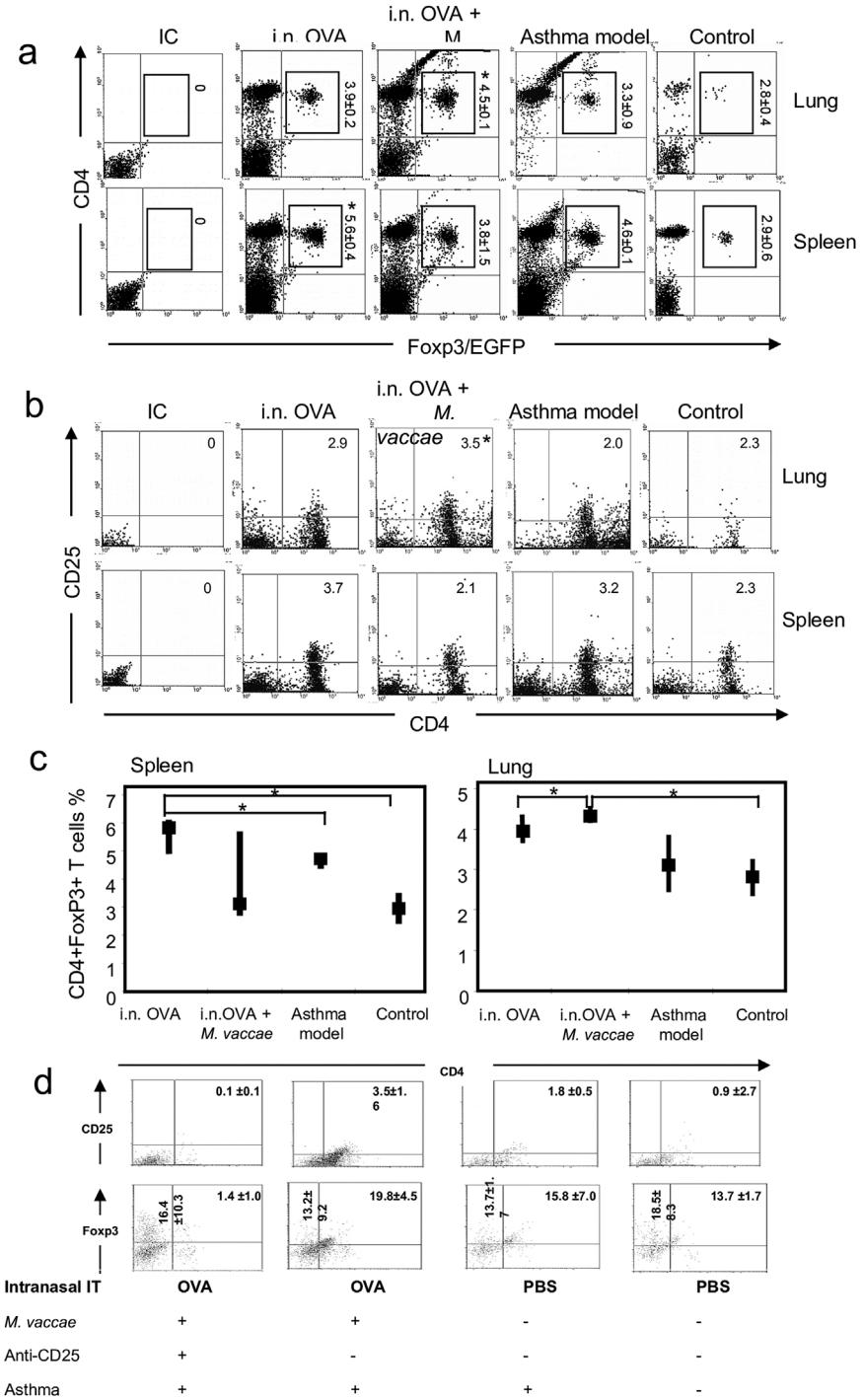

Fig. 2. Flow cytometric analysis of splenocytes and lung lymphocytes. (a) $CD4^{+}FoxP3^{+EGFP+}$ T regulatory cells were assessed by flow cytometry. The proportion of $CD4^{+}FoxP3^{+EGFP+}$ T regulatory cells is significantly greater in the IN-OVA + *M. vaccae* than the asthma and control groups ($P < 0.05$). The IN-OVA therapy group tend to have a greater proportion of $CD4^{+}FoxP3^{+EGFP+}$ T regulatory cells in lymphocytes and have a significantly greater proportion in splenocytes than the asthma and control groups ($P < 0.05$). (b) $CD4^{+}CD25^{+}$ T cells were assessed by flow cytometry. Similar results were found for $CD4^{+}CD25^{+}$ T cells, the proportion of which is significantly greater in the IN-OVA + *M. vaccae* group in lung lymphocytes ($P < 0.05$) and tends to be greater in splenocytes in the IN-OVA group than in mice with asthma. (c) Amounts of CD4 + FoxP3 + T regulatory cells in spleen and lung are shown. (d) CD4 + CD25+ and CD4 + FoxP3 + T regulatory cells in lung lymphocytes and splenocytes were compared between groups. Depletion of $CD25^{+}$ T cells before IN-OVA + *M. vaccae* administration led to significantly fewer $CD4^{+}CD25^{+}$ and $CD4^{+}$ $Foxp3^{+}$ T cells than in the IN-OVA + *M. vaccae* group ($P = 0.01$ and $P = 0.01$, respectively), asthma group ($P = 0.008$ and $P = 0.01$ respectively), and controls ($P = 0.03$ and $P = 0.009$, respectively). IT, immunotherapy.

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RESULTS

OVA immunotherapy increases the proportion of $CD4^+CD25^+$ $Foxp3^{+EGFP+}$ T cells in the lung and spleen

In both the IN-OVA and IN-OVA + *M. vaccae* groups, the proportion of $CD4^+$ $Foxp3^{+EGFP+}$ T cells in the lung was greater than in the asthma model group (3.9 ± 0.2%, 4.5 ± 0.1% and 3.3 ± 0.9%, respectively) (Fig. 2 a,c). The proportion was significantly higher in the adjuvant group than in both controls and the IN-OVA alone group ($P < 0.05$). Spleen $CD4^+$ $Foxp3^{+EGFP+}$ T cells comprised a greater proportion in the IN-OVA group (5.6 ± 0.4%) than in both controls or the asthma groups (2.9 ± 0.6% and 4.6 ± 0.1%, respectively, $P < 0.05$) (Fig. 2 a,c).

The same trends were observed for $CD4^+CD25^+$ T cells in the lung and spleen of mice. $CD4^+CD25^+$ T cells comprised a significantly greater proportion of lung lymphocytes in the IN-OVA + *M. vaccae* group ($P < 0.05$) and tended to be greater in splenocytes in the IN-OVA group than in the asthma and control groups (Fig. 2b).

Depletion of $CD25^+$ T cells before IN-OVA + *M. vaccae* administration led to significantly smaller proportions of $CD4^+CD25^+$ and $CD4^+$ $Foxp3^+$ T cells than in the IN-OVA + *M. vaccae* group ($P = 0.01$ and $P = 0.01$, respectively), asthma group ($P = 0.008$ and $P = 0.01$ respectively) and controls ($P = 0.03$ and $P = 0.009$, respectively) (Fig. 2d).

OVA immunotherapy increases IFN-γ- and IL-10-secreting T cells among splenocytes

A cytokine intracellular secretion assay was used to investigate cytokine secretion from OVA-induced T cells from splenocytes. Amounts of OVA-induced IFN-γ and IL-10 were found to be higher in the IN-OVA + *M. vaccae* group than in the other groups. Interestingly, those cells were found to be Foxp3 negative in the spleen (Fig. 3a,b).

Amounts of IFN-γ and IL-10 are greater and of IL-5 smaller with OVA immunotherapy

It was found that amounts of OVA-induced IFN-γ were higher in the IN-OVA + *M. vaccae* group both in spleen and lung than in the IN-OVA group ($P < 0.05$) (Fig. 4a). Likewise, when T cells from lung and spleen were isolated and stimulated with plate-bound anti-CD3, secretion of IFN-γ was found to be highest in the

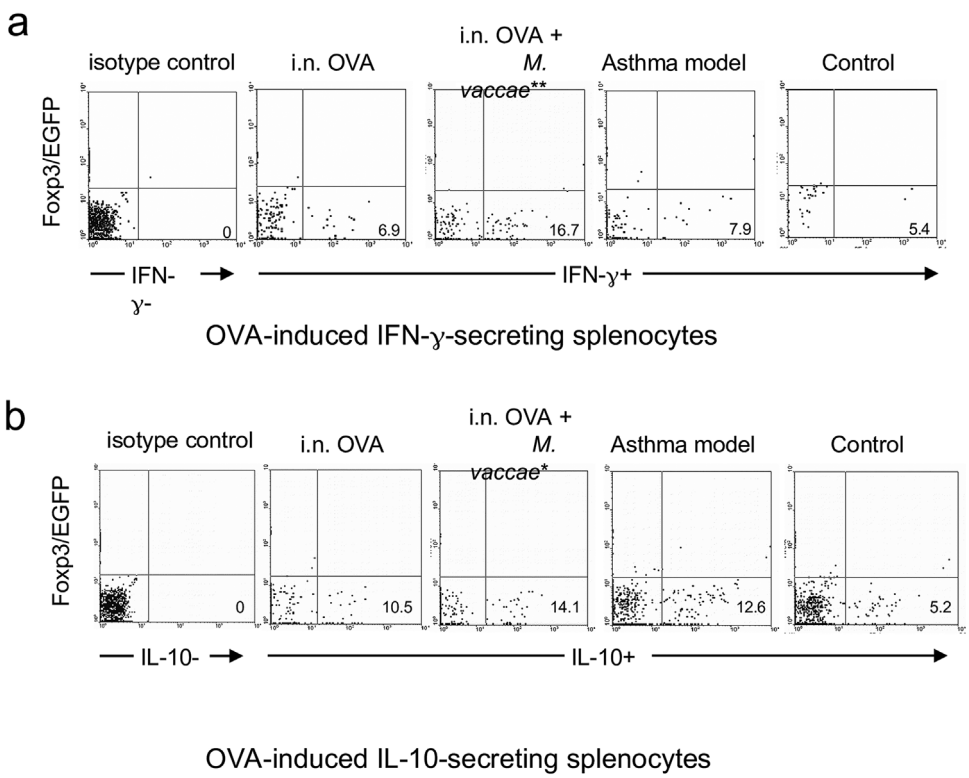

Fig. 3. Amounts of (a) IFN-γ- and (b) IL-10-secreting cells among OVA-induced splenocytes. Splenocytes from all groups were stimulated with OVA for 16 hr *in vitro*, after which IFN-γ- and IL-10-secreting T cells were analyzed by flow cytometry. * $P < 0.05$; ** $P < 0.01$.

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IN-OVA + *M. vaccae* group ($P < 0.05$) (Fig. 4b). Amounts of *M. vaccae*-induced IFN-γ were higher in both the lung and spleen from the IN-OVA group than in those from the other groups.

Greater amounts of OVA-induced IL-10 were detected in lung from the IN-OVA + *M. vaccae* group than in that from the IN-OVA alone group ($P < 0.05$). In addition, both IN-OVA and IN-OVA + *M. vaccae*

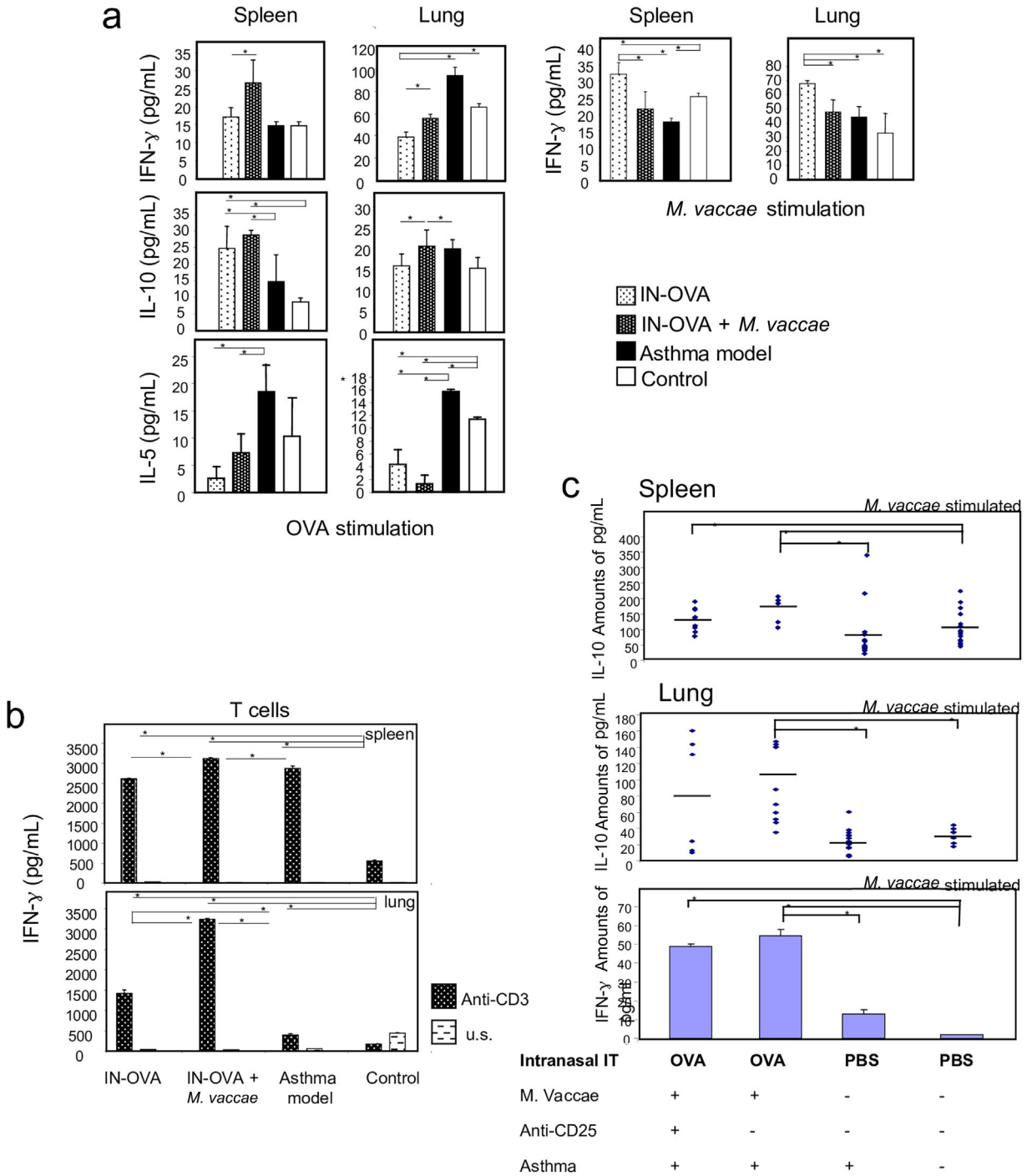

Fig. 4. Amounts of cytokines. (a) OVA and *M. vaccae* stimulated splenocyte and lung lymphocyte culture supernatants were collected and IL-10, IL-5 and IFN-γ assessed by ELISA. * $P < 0.05$. (b) Amounts of IFN-γ of plate-bound anti-CD3-stimulated and unstimulated T cells. (c) The effect of *in vivo* CD-25 neutralization on cytokine profiles. * $P < 0.05$. [Color figure can be viewed at wileyonlinelibrary.com]

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treated groups had more IL-10 in spleen than the asthma model group and controls (Fig. 4b).

Significantly smaller amounts of OVA-induced IL-5 were detected in both spleen and lung lymphocytes in the IN-OVA and IN-OVA + *M. vaccae* groups than in those from the asthma model group (Fig. 4a).

Significantly greater amounts of both IL-10 and IFN-γ were detected in lung and spleen from the IN-OVA + *M. vaccae* group than in those from the asthma group ($P < 0.05$). This difference disappeared after *in vivo* depletion of $CD25^+$ T cells (Fig. 4c).

Amounts of OVA-specific IgG1 and IgE were smaller with OVA immunotherapy

Amounts of OVA-specific IgG1 were greatest in the asthma model group, the IN-OVA + *M. vaccae* group having significantly less; thus difference was more pronounced after $CD25^+$ T cell depletion ($P < 0.05$) (Fig. 5a). Although there was a trend to smaller amounts of OVA-specific IgE in theIN-OVA + *M. vaccae* treatment group, this difference was not statistically significant (Fig. 5b).

Intranasal OVA immunotherapy improves remodeling in airways

The experimental asthma model group had thicker epithelium and smooth muscle in small ($P = 0.001$) and medium ($P = 0.002$ and $P = 0.019$, respectively) airways than did the control group (Fig. 6a,b), whereas the IN-OVA group showed less thick smooth muscle in medium airways ($P < 0.05$) than the asthma model group ($P < 0.05$). The IN-OVA + *M. vaccae* group had less thick smooth muscle and epithelium in large and small airways than the asthma model group. Moreover, there were no statistically significant differences in smooth muscle thickness of small, medium or large airways between either the IN-OVA or IN-OVA + *M. vaccae* group and controls. There were no statistically significant differences in thickness of epithelium between the IN-OVA + *M. vaccae* group and controls in all sized airways, whereas in the IN-OVA group the thickness was comparable to that of medium and large airways of controls only.

DISCUSSION

Several studies have shown that, although CD25 expression is important for differentiating T cells and regulatory T cells, co-expression of Foxp3 points directly to the presence of regulatory T cells (8). Clinical studies of grass pollen immunotherapy have also revealed more abundant $CD4^+CD25^+$ $Foxp3^{+EGFP+}$ T cells in the nasal mucosa than in untreated patients with hay fever (9).

Recent studies have shown that specific immunotherapy can be improved by co-administration of adjuvants (10). A combination of antigen and adjuvant molecules can influence antigen-stimulated T cell cytokine profiles (11). Studies have demonstrated an inverse correlation between Th1-type hypersensitivity to *M. tuberculosis* and atopy in Japanese school children, and heat-killed or live *Mycobacteria* down-regulate Th2 responses to allergens (12, 13). Consistent with this finding, another study has investigated using preparations of non-pathogenic *M. vaccae* in prophylactic treatment of allergic inflammation and asthma in murine models (14). In addition, it has previously been demonstrated that migration of Tregs to allergic lung tissue and lymph nodes implicates the expression of chemokine receptors CCR4 and CCR7, respectively (15). A study has shown that CCL17 and CCL22, both ligands for CCR4, have roles in the migration and homing of $CD4^+CD25^+$ Tregs to airway tissues after allergen responses (16). An interesting finding of our study is that intranasal OVA immunotherapy alone results in accumulation of $CD4^+CD25^+$ T and $CD4^+$ $Foxp3^{+EGFP+}$ T cells in the spleen and that addition of *M. vaccae* as adjuvant increases such accumulation in the lung. These results may indicate the importance of using adjuvants along with mucosal immunotherapy to promote migration of Treg cells to end organs.

It has been shown that intranasal immunotherapy with Bet-v-1 induces long-term induction of tolerance with enhanced expression of IL-10, TGF-β and Foxp3-mRNA

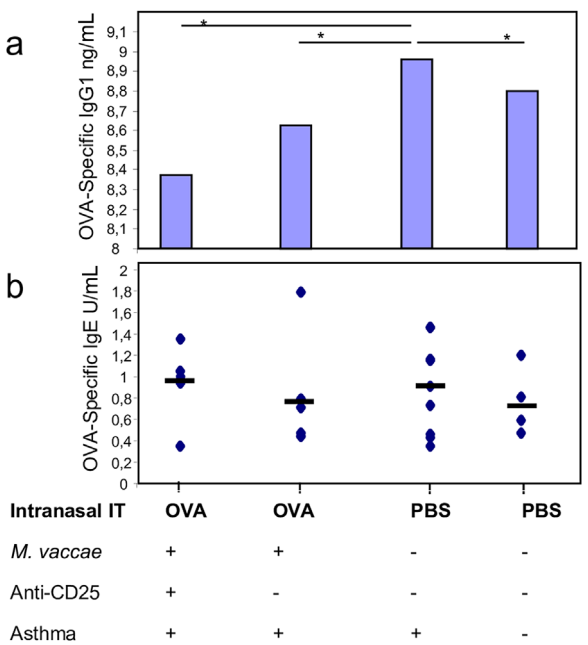

Fig. 5. Amounts of OVA-specific IgG1 and IgE. [Color figure can be viewed at wileyonlinelibrary.com]

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expression in $CD4^+$ T cells (17). In contrast, after allergen-specific immunotherapy two different subsets of Treg cells, namely Foxp3-expressing adaptive Tregs and IL-10 producing- $Foxp3^-$ $CD25^+$ Tr1 cells, reportedly coexist (18). In the present study, we showed that intranasal OVA immunotherapy along with *M. vaccae* immunization enhances both $Foxp3^-$ IL-10-secreting and $Foxp3^-$ IFN-γ secreting T cells (that is, Tr1 type regulatory cells) in the spleen together with accumulation of $CD4^+CD25^+Foxp3^+$ T cells in the lung. In contrast, we found that intranasal OVA immunotherapy alone results in preferential accumulation of $CD4^+CD25^+$ $Foxp3^{+EGFP+}$ T type regulatory cells in the spleen without any IL-10 and IFN-γ-secreting $Foxp3^-$ T cells.

In the current study, both immunotherapy protocols investigated suppressed IL-5 secretion in a murine asthma model. Amounts of OVA-stimulated IL-10 from lung and spleen lymphocytes were greater in the adjuvant group; in contrast, they were greater in splenocytes only in the IN-OVA immunotherapy group. OVA stimulated IFN-γ

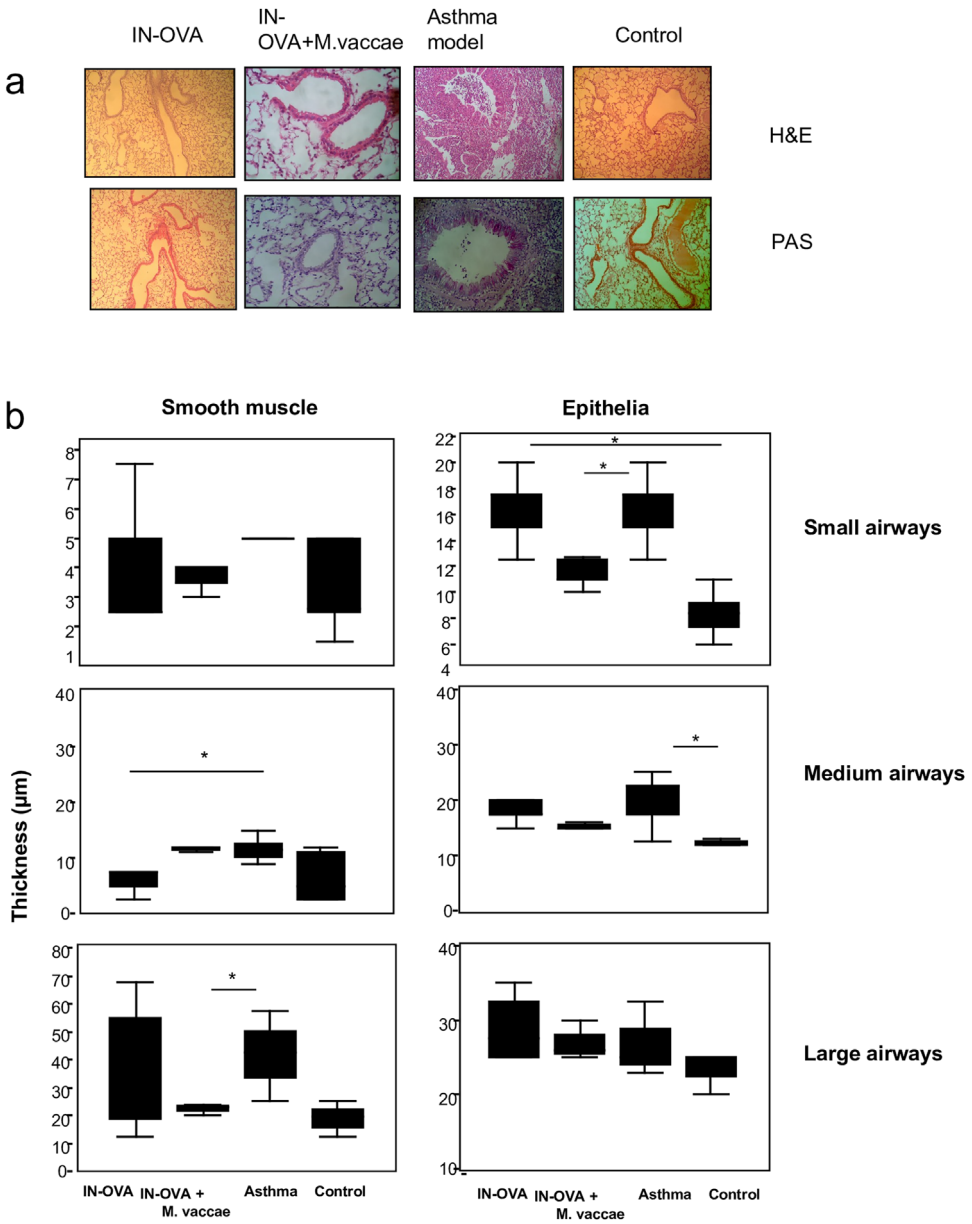

Fig. 6. Hematoxylin and eosin- and periodic acid–Schiff-stained lung sections for all groups . **(40**×). (b) Statistical analysis of smooth muscle and epithelia thickness of airways showed that large airway smooth muscle thickness was lowered in IN-OVA + *M. vaccae* group. H&E, hematoxylin and eosin; PAS, periodic acid–Schiff. [Color figure can be viewed at wileyonlinelibrary.com]

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secretion from spleen lymphocytes was greater only in the IN-OVA immunotherapy + *M. vaccae* group. Upon anti-CD3 stimulation the adjuvant group demonstrated greater amounts of IFN-γ than in all other groups.

In addition, the small amounts of both OVA-specific IgG1 and IgE observed in this group further indicate successful down-regulation of Th2 responses with antigen-specific immunotherapy along with a bacterial adjuvant.

Both the intranasal immunotherapy and adjuvant groups demonstrated amelioration of the histopathology of asthma when compared to controls, which is in accordance with our finding concerning preventive strategies for asthma (4).

T cells with regulatory activity secrete anti-inflammatory cytokines IL-10 and TGF-β, which play crucial roles in the induction of tolerance in allergy (17, 19). Nevertheless, the main role of $CD4^{+}CD25^{+}$ and $CD4^{+}CD25^{-}$ T regulatory cells in tolerance induction in therapeutic and prophylactic murine models of asthma is still a promising research area. In the present study, we showed that depletion of $CD4^{+}CD25^{+}$ T cells with *in vivo* anti-CD25mb PC61 led to weaker Foxp3 expression and smaller amounts of IL-10 and IFN-γ in the mucosal allergen immunotherapy plus *M. vaccae* group. Such impaired function in the $CD25^{+}$T cell depleted model has also been detected in a therapeutic approach model using intranasal treatment (20).

We found that intranasal allergen-specific immunotherapy along with *M. vaccae* immunization enhances the proportion of $CD4^{+}CD25^{+}$ $Foxp3^{+\mathbf{EGFP+}}$ T cells in the target organ, lung, in a murine model of asthma. In contrast, intranasal OVA immunotherapy without the adjuvant, *M. vaccae*, resulted in a greater proportion of $CD4^{+}CD25^{+}$ $Foxp3^{+\mathbf{EGFP+}}$ T cells in spleen.

In conclusion, in this study we found that mucosal administration of OVA antigen with *M. vaccae* prior to establishment of an asthma model results in a shift of $CD4^{+}CD25^{+}$ $Foxp3^{+\mathbf{EGFP+}}$ cells from spleen to the end organ exposed to antigen challenge in $Foxp3^{+\mathbf{EGFP+}}$ bicistronic reporter mice.

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DISCLOSURE

The authors have no conflicts of interest to disclose.

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