

ORIGINAL ARTICLE

CD4⁺CD25⁺ T cell depletion impairs tolerance induction in a murine model of asthma

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Summary

Background Regulatory T cells (Tregs) are key players in controlling the development of airway inflammation. However, their role in the mechanisms leading to tolerance in established allergic asthma is unclear.

Objective To examine the role of Tregs in tolerance induction in a murine model of asthma.

Methods Ovalbumin (OVA) sensitized asthmatic mice were depleted or not of CD25⁺ T cells by anti-CD25 PC61 monoclonal antibody (mAb) before intranasal treatment (INT) with OVA, then challenged with OVA aerosol. To further evaluate the respective regulatory activity of CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells, both T cell subsets were transferred from tolerized or non-tolerized animals to asthmatic recipients. Bronchoalveolar lavage fluid (BALF), T cell proliferation and cytokine secretion were examined.

Results Intranasal treatment with OVA led to increased levels of IL-10, TGF-β and IL-17 in lung homogenates, inhibition of eosinophil recruitment into the BALF and antigen specific T cell hyporesponsiveness. CD4⁺CD25⁺Foxp3⁺ T cells were markedly upregulated in lungs and suppressed *in vitro* and *in vivo* OVA-specific T cell responses. Depletion of CD25⁺ cells before OVA INT severely hampered tolerance induction as indicated by a strong recruitment of eosinophils into BALF and a vigorous T cell response to OVA upon challenge. However, the transfer of CD4⁺CD25⁻ T cells not only suppressed antigen specific T cell responsiveness but also significantly reduced eosinophil recruitment as opposed to CD4⁺CD25⁺ T cells. As compared with control mice, a significantly higher proportion of CD4⁺CD25⁻ T cells from OVA treated mice expressed mTGF-β.

Conclusion Both CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells appear to be essential to tolerance induction. The relationship between both subsets and the mechanisms of their regulatory activity will have to be further analyzed.

Keywords allergy, asthma, IL-17, regulatory T cells, TGF-β, tolerance

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Introduction

Asthma is a complex inflammatory disorder of the airways caused by environmental factors in genetically predisposed individuals (atopic) and non-specifically triggered by irritants or infections. In susceptible individuals, airways inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness and cough. Asthma prevalence has dramatically increased over the past two decades [1, 2]. In allergen specific immunotherapy of asthma, it was first assumed that T helper type-1 (Th1) cells had a beneficial role by down-regulating Th2 cells [3, 4]. However, as a greater understanding of Th1 cells developed, it appeared that Th1-associated inflam-

matory response could exacerbate allergic airways inflammation [5]. This suggested that other forms of immunity than pure Th1/Th2 responses must down-regulate the development of asthma and allergic diseases [6]. There are strong evidences for implication of CD4⁺CD25⁺ regulatory T cells in controlling allergic disease. They represent 5–10% of CD4⁺ T cells and suppress Th1-mediated diseases but their role in Th2 diseases is a more recent point of interest and still debatable [7–9]. Some studies showed little or no effect of CD4⁺CD25⁺ T cells depletion on eosinophil recruitment in allergic mice [9, 10]. In contrast, the transfer of CD4⁺CD25⁺ T cells to sensitized mice reduced airway hyperresponsiveness (AHR), eosinophils recruitment and Th2 cytokine

expression in the lung in an IL-10 dependent way [8, 11]. Other regulatory T cell subsets (mainly Tr1-like and Th3 cells) can also suppress Th2 pathologies by production of IL-10 and TGF- β . They were highly efficient in down-regulating allergen-induced AHR in previously sensitized mice [12].

In the present study, and in view of previous findings [7, 13], we evaluated the respective role of CD4⁺CD25⁺ and CD4⁺CD25⁻ regulatory T cells in the induction of tolerance in a mouse model of asthma established in our laboratory (C. Pellaton et al., unpublished data). In this model, tolerance was characterized by inhibition of eosinophils recruitment into the lungs, allergen specific T cell hyporesponsiveness, inhibition of Th2 cytokine secretion and diminished AHR. We here show that depletion of CD25⁺ T cells impaired allergen specific tolerance induced by nasal application of ovalbumin (OVA). Nonetheless, whereas the transfer of CD4⁺CD25⁺ T cell subset to asthmatic recipient was unable to significantly decrease eosinophil recruitment into bronchoalveolar lavage fluid (BALF) in this non-transgenic model, the transfer of CD4⁺CD25⁻ T cells from OVA-tolerized animals markedly decreased both lung eosinophils infiltrates and OVA specific T cell response. Taken together, our study suggests that CD4⁺CD25⁺ T cells may play a key role in the induction of CD4⁺CD25⁻ T cells with regulatory properties.

Materials and methods

Animals

Four-week-old female BALB/c mice (Harlan, AD Horst, the Netherlands) were maintained under standard housing conditions and used at the age of 6–8 weeks (authorization number 1044.4 from the Service Vétérinaire du Canton de Vaud).

Allergic asthma induction and tolerization

Mice were sensitized intraperitoneally (i.p.) at days 0/14 with 10 μ g of OVA (Fluka, Buchs, Switzerland, LPS < 15 pg/mg) coupled to 1 mg alum (Sigma, Buchs, Switzerland). On days 24/26/28, animals were exposed to 0.25% OVA aerosol generated by a nebulizer (De Vilbiss, Malsch, Germany) during 20 min. Mice received intranasally (i.n.) a 20 μ L drop containing 1.5 mg OVA or phosphate-buffered saline (PBS) on days 35/36/37 under light anesthesia with halothane. Mice were challenged by 0.25% OVA aerosol on days 45/47/49. For intranasal treatment (INT), a stock solution of 75 mg/mL OVA was prepared from a 100 mg/mL OVA solution prepared in PBS, centrifuged at 4000 g for 30 min and passed through a 22 μ m filter (Nalgen, Roskilde, Denmark). Preparation was adjusted to 75 mg/mL after colorimetric assay (BC Assay protein dosage kit, Uptima, Montluçon, France).

LPS content was determined using *Limulus* Amebocyte Lysate Assay (LAL, Charles River Endosafe, Charleston, SC, USA) according to manufacturer's protocol.

Anti-CD25 monoclonal antibody PC61 and CD25⁺ T cells depletion

Anti-mouse CD25 mAb PC61 (rat IgG1, kind gift from Dr A. Wilson, Ludwig Institute, Epalinges, Switzerland) was purified from culture supernatant on protein G sepharose column. Intraperitoneal treatment with 1 mg PC61 yielded more than 80% CD4⁺CD25⁺ T cells depletion in PBMC and spleen during 7 days. Mice were treated i.p. with 1 mg PC61 at days 31, 38 and 45 (long-term depletion) or only at day 31 (short-term depletion) (Fig. 4A). These mice were defined as OVA INT/PC61 (OVA INT and PC61 treatment) or PBS INT/PC61 (PBS INT and PC61 treatment). Because preliminary experiments demonstrated similar and reproducible results between groups of mice treated with 1 mg rat IgG1 isotype control or with PBS, we limited the control groups to PBS injection only in further experiments.

Cell preparation

Lung tissue. Lungs were minced in NaCl 0.9% and incubated with collagenase (0.5 mg/mL), DNase (0.1 μ g/mL) and Ca²⁺ (5 mM). Cells were isolated using a Dounce homogenizator and filtered on 40 μ m nylon cell strainer (BD Falcon, Basel, USA).

Draining lymph nodes. Bronchial lymph nodes (BLN) were harvested in Dulbecco's modified Eagle's medium and homogenized on a 40 μ m nylon cell strainer. Red blood cells were lysed and cells were counted.

Flow cytometry

Cells were incubated with anti-CD4 fluorescein isothiocyanate (L3T4, BD Pharmingen, San Diego, CA, USA), anti-CD25 APC (PC61, BD Pharmingen) and TGF- β PE (IQ Products, Groningen, the Netherlands) according to manufacturer's protocol.

Foxp3 intracellular staining was performed using anti-mouse/rat Foxp3 staining set (FJK-16s, eBioscience, San Diego, CA, USA) according to manufacturer's protocol. For IL-10, lungs cells were cultured overnight in DMEMc, 500 μ g/mL OVA and 10 μ g/mL Brefeldin A (Sigma) was added during the last 4 h of culture. Cells were then stained with anti-IL-10 APC (JES5-16E3, BD Pharmingen) using Foxp3 staining protocol from eBioscience. Flow cytometry acquisitions were performed on a FACScalibur (BD Biosciences) and analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

Isolation of CD4⁺CD25⁺ and CD4⁺CD25⁻ cells

Lungs and bronchial lymph nodes CD4⁺ T cells were enriched using a BDTM Imag Mouse CD4 T lymphocyte enrichment set according to the manufacturer's protocol (BD Pharmingen). CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells were then purified using MACS according to the manufacturer's protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). CD4⁺CD25⁺ T cells were positively selected and CD4⁺CD25⁻ T cells were negatively selected in the same purification process. Purity was typically >85%.

Adoptive cell transfer

Bronchial lymph nodes and lungs were collected at day 52 from BALB/c donor mice. 5×10^5 CD4⁺CD25⁺ or CD4⁺CD25⁻ T cells in 100 μ L NaCl 0.9% were intravenously injected in recipient BALB/c asthmatic mice (day 35). Control recipients mice received NaCl 0.9% alone. At days 36/38/40, recipient mice were challenged with OVA 0.25% aerosol for 20 min and killed at day 43 (Fig. 5A).

Proliferation assay

Cells (1×10^5) were cultured as previously described [14] with 50 μ g/mL OVA. For co-cultures, 1×10^5 CD4⁺CD25⁻ cells and increasing numbers of CD4⁺CD25⁺ cells were cultured in the presence of 5×10^4 peritoneal macrophages from peritoneal cavity of donor mice. After 3 days of culture, 1 μ Ci [³H]-Thymidine (Hartmann Analytic GmbH, Braunschweig, Germany) per well was added for the last 18 h of culture. Stimulation indexes represent the ratio of OVA-stimulated vs. non-stimulated cells.

Bronchoalveolar lavage fluid and cell count

Animals were anesthetized with 5 μ g thiopental. Trachea was cannulated and lungs washed six times with 500 μ L PBS was injected into the lung. Bronchoalveolar lavage fluid was immediately stored on ice and cells counted. After centrifugation on glass plates (Cytospin, Thermo Fisher Scientific, Waltham, MA, USA) and staining with DiffQuik (Medion diagnostics, Düringen, Switzerland) a differential count of 200 cells was performed using standard morphological criteria.

Quantification of serum antibodies and lungs cytokines by enzyme-linked immunosorbent assay

For serum antibodies, 96-well Nunc Maxisorp immunoplates (Life Technologies, Basel, Switzerland) were coated with 5 μ g/mL OVA for direct IgE, IgG1 and IgG2a. Paired antibodies for IgG1, IgG2a (Caltag, Basel, Switzerland) and direct IgE measurement (BD Pharmingen) were used in standardized sandwich ELISAs according to the manufacturer's protocol. Purified IgE (BD Pharmingen) was used as standard. For indirect IgE measurement, plates

were coated with 2 μ g/mL rat anti-mouse IgE (BD Pharmingen), incubated with 500 ng/mL of biotinylated OVA and revealed with streptavidine labelled alkaline phosphatase (Sigma-Aldrich, Buchs, Germany).

For lungs cytokines, lungs were collected at day 52, snap-frozen on dry ice and homogenized in PBS containing proteases inhibitors (Roche, Basel, Switzerland). Cytokines TGF- β and IL-17A were measured by ELISA according to manufacturer's protocols (BD OptEIATM Set Human TGF- β 1, BD Pharmingen and DuoSet ELISA mouse IL-17A, R&D Systems, Abingdon, UK). For IL-10 measurement, plates were coated with a capture anti-mouse IL-10 mAb (JES052A5, R&D Systems). Lung homogenates were incubated 2 h at room temperature. A secondary biotinylated anti-mouse IL-10 mAb (JES052A5, R&D Systems) was added and the presence of IL-10 was revealed using Extravidin-Alkaline-phosphatase and its substrate (Sigma). Recombinant mouse IL-10 from (BD Pharmingen) was used as a standard.

Statistical analysis

All results are expressed as mean $\pm$ SD. Statistical significance between groups was tested using a Student *t* test. *P*-value <0.05 was considered significant (*). Graph generation and statistical analysis were performed by using Prism software (version 4; GraphPad, La Jolla, CA, USA).

Results

Depletion of CD4⁺CD25⁺ T cells leads to airway inflammation

To examine the role of CD4⁺CD25⁺ T cells in the induction of tolerance, we studied BALB/c mice in a therapeutic asthma model based on three phases including appropriate controls (Fig. 1A): mice were first sensitized to OVA i.p., then exposed to OVA aerosols 0.25% (asthma induction phase). In a second phase, OVA was administered i.n. to induce tolerance (INT phase) and finally mice were re-exposed to OVA aerosols 0.25% (allergen challenge phase). After the asthma induction phase, mice were depleted of CD25⁺ T cells by injecting anti-CD25 (PC61) mAb at days 31, 38 and 45 (Fig. 1A). These injections depleted >80% of CD4⁺CD25⁺ T cells and >95% of CD4⁺CD25⁺Foxp3⁺ T cells in the BLN (data not shown). Differential cell counts in BALF were performed at two time-points: (1) at day 43 (6 days after INT) to obtain an early picture of the immune response post-INT with OVA, (2) at day 52 (3 days after allergen challenge) to analyze the ability of INT with OVA to induce tolerance to the allergen (Fig. 1B). At day 43, PC61 treatment did not induce significant difference in the relative proportion of macrophages, lymphocytes and eosinophils. In contrast, after OVA aerosols challenge (day 52), the proportion and

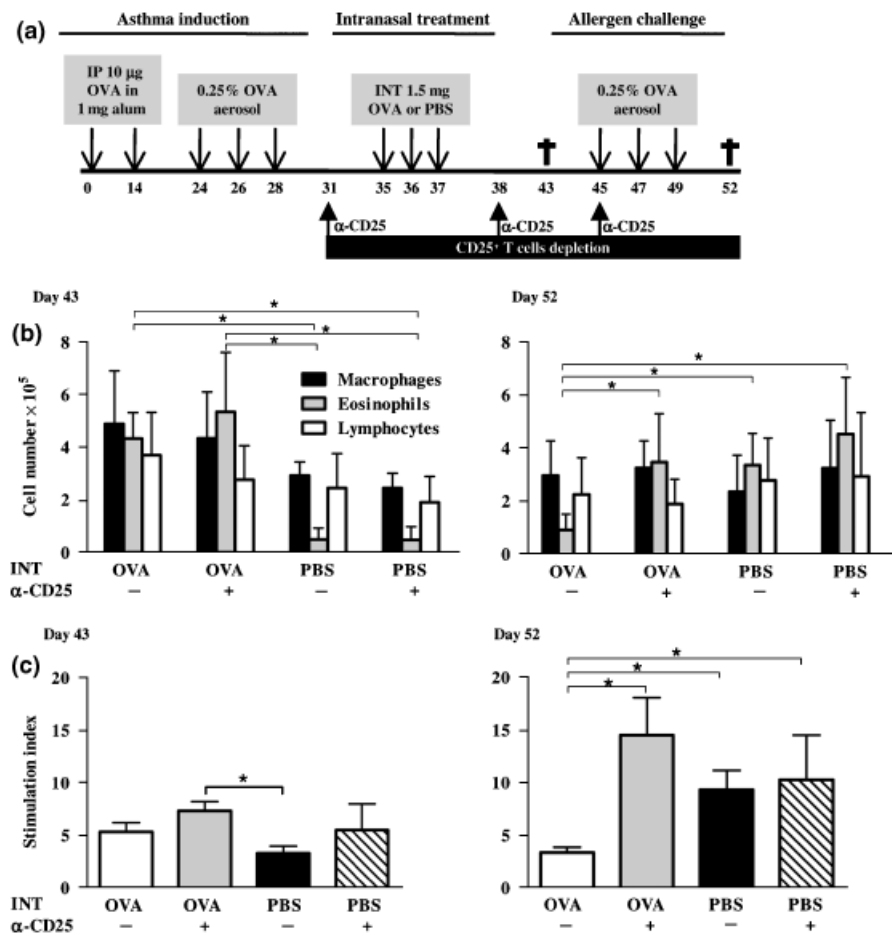


Fig. 1. CD4⁺CD25⁺ T cells depletion leads to eosinophil recruitment into BALF and loss of T cell hyporesponsiveness to OVA. (a) Long-term depletion protocol. Briefly, BALB/c mice were sensitized and exposed to OVA aerosols (asthma induction phase). Eight days after the last aerosol, mice were treated intranasally every day for 3 days with 1.5 mg of OVA or with PBS (intranasal treatment phase). At days 45/47/49, mice were challenged with OVA aerosol (allergen challenge phase). Mice received i.p. injections of PBS or PC61 (1 mg) at days 31/38/45 and were killed at day 43 or 52. (b) Eosinophils, macrophages and lymphocytes in BALF were counted at days 43 and 52. (c) BLN cells were cultured 4 days with OVA and proliferation was measured using [³H]-thymidine incorporation. *In vitro* proliferation assays were performed with individual lymph nodes and results are expressed as mean ± SD of 4–8 mice per group (**P* < 0.05). BALF, bronchoalveolar lavage fluid; BLN, bronchial lymph nodes; OVA, ovalbumin; PBS, phosphate-buffered saline.

absolute number of eosinophils in BALF from OVA INT/PC61 mice was significantly enhanced as compared with non-depleted OVA INT mice (respectively, 39% and 15% eosinophils), and did not differ from PBS INT and PBS INT/PC61 controls (respectively, 40% and 43% eosinophils). Pretreatment with PC61 mAb appeared thus to impair tolerance induced by OVA INT. These data suggest that CD4⁺CD25⁺ T cells may play a key role in the mechanisms leading to inhibition of inflammatory cell recruitment (mainly eosinophils) into BALF after OVA aerosols challenge.

Depletion of CD4⁺CD25⁺ T cells abrogates T cell hyporesponsiveness to ovalbumin

At day 43 (Fig. 1C, left panel), BLN cells from OVA INT/PC61 mice proliferated more vigorously than PBS INT controls (SI = 7.3 ± 1.5 vs. 3.3 ± 1.5) in response to OVA,

although proliferation was comparable between OVA INT/PC61 and PBS INT/PC61 animals at this time-point. At day 52 (Fig. 1C, right panel), OVA INT mice cells responded poorly to OVA whereas OVA INT/PC61 strongly proliferated (SI = 3.4 ± 1.4 vs. 14.5 ± 8.8). In contrast, CD25 depletion had no significant effect on BLN cell proliferation in PBS INT groups. Globally, these data indicate that CD25⁺ T cells depletion led to an increase in T cell proliferation to OVA after challenge compatible with decreased T cell suppressive activity.

CD4⁺CD25⁺ T cell depletion inhibits ovalbumin specific IgG2a production

After challenge with OVA aerosols (day 52), there was no significant difference in serum level of OVA specific IgE between depleted and non-depleted mice (Fig. 2A). For comparison, a control group of mice sensitized to OVA and

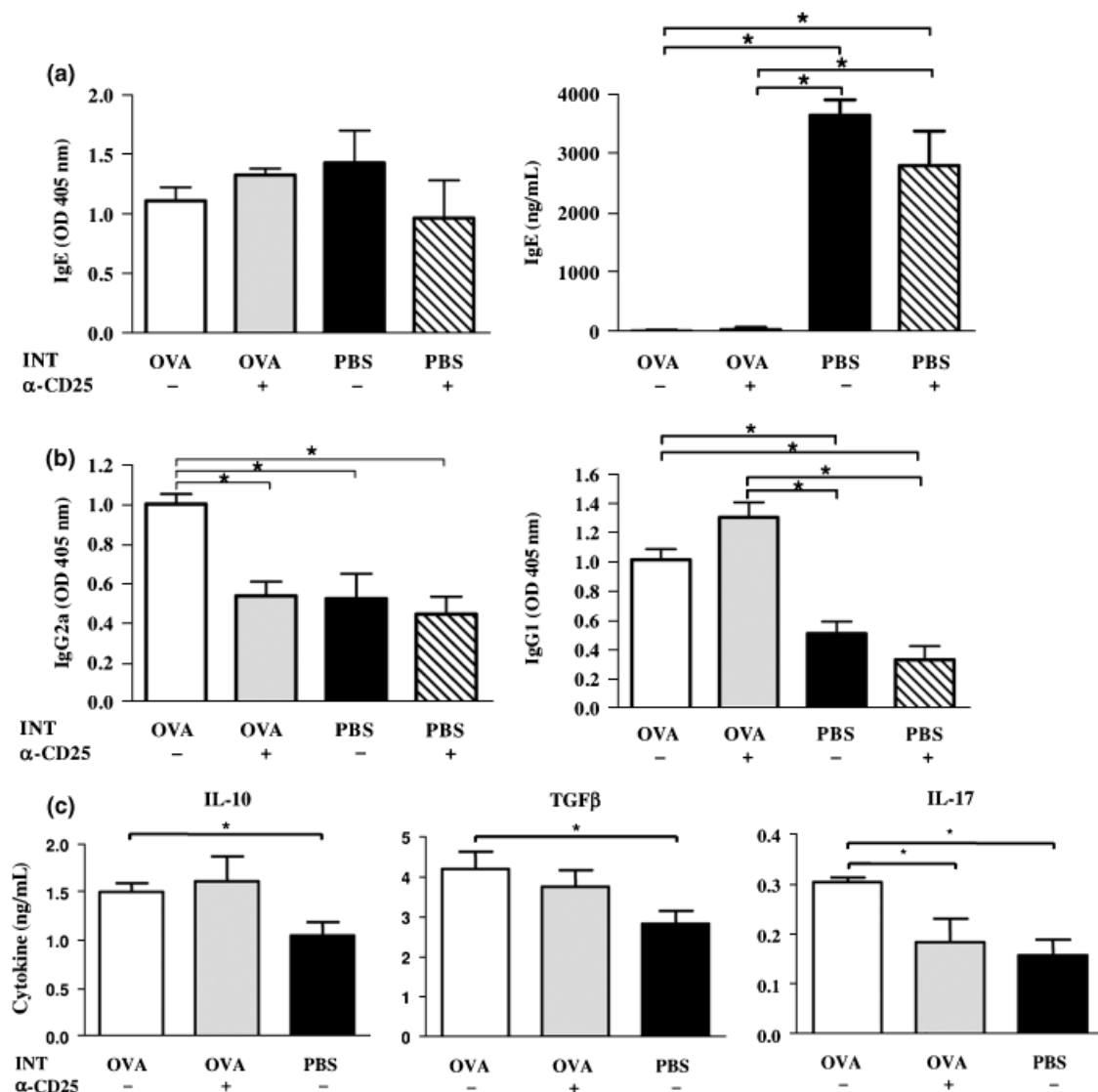


Fig. 2. CD4⁺CD25⁺ T cell depletion down-regulates anti-OVA IgG2a and decreases IL-17 in lung homogenates. Lungs and sera were collected at day 52 (a) Serum OVA specific IgE (indirect ELISA left panel, direct ELISA right panel) (b) Serum IgG2a and IgG1 (c) IL-10, TGF-β and IL-17 were measured by ELISA in lung homogenates. Results are expressed as mean of 4–5 mice/group (* $P < 0.05$). OVA, ovalbumin.

treated i.n. with PBS before aerosol challenge with PBS typically displayed baseline mean serum level of 640 ± 149 ng/mL of OVA-specific IgE (data not shown). We compared two ELISA protocols to detect OVA-specific IgE (Fig. 2A, left and right panel): a direct method (coating with OVA, incubation with sera and revelation with alkaline phosphatase labeled anti-IgE) and an indirect method (coating with anti-IgE, incubation with sera and revelation with biotinylated OVA). Comparison of the two ELISA results strongly suggested that INT with OVA induced *de novo* competitive OVA specific IgG able to inhibit IgE binding to OVA (Fig. 2A, right panel). Induction of competitive antibodies was not influenced by CD25⁺ cell depletion. This phenomenon was previously observed in human desensitization to allergen and plays a key role in protection upon allergen re-exposure [15]. Furthermore a

significant reduction in OVA specific IgG2a production was observed in CD25⁺ cells depleted animals (Fig. 2B, left panel) ($P < 0.05$). At the same time-point (Fig. 2B, right panel), depleted or non-depleted OVA INT mice displayed enhanced IgG1 serum levels ($P < 0.05$) as compared with PBS INT groups. Taken together, our results suggest that CD4⁺CD25⁺ T cell depletion did not influence antigen-specific IgE and IgG1 production in mice treated i.n. with OVA. In contrast the Th1 type IgG2a production was strongly diminished by CD25 depletion.

CD4⁺CD25⁺ T cells depletion diminished interleukin-17 secretion in the lungs

IL-10, TGF-β and IL-17 levels were measured by ELISA in lung homogenates from lungs harvested at day 52

(Fig. 2C). IL-10 and TGF- β were produced in higher quantity in lungs of tolerized mice (OVA INT) than in non-tolerized mice (PBS INT). However, there was no significant change after treatment with PC61, suggesting that the major source of IL-10 and TGF- β in lungs may not be the CD25⁺ T cells. In contrast, anti-CD25 treatment significantly decreased IL-17 production in the lungs of OVA INT mice. In lung homogenates of PBS INT/PC61 control mice, levels of IL-10 and IL-17 were comparable to PBS INT mice (not shown).

Characterization of potential regulatory T cell subsets

Because CD25⁺ T cells appeared essential in the induction of tolerance, we followed CD4⁺CD25⁺Foxp3⁺ regulatory T cells in the lungs using flow cytometry (Fig. 3A). At days 0 and 21, respectively, 0.45% and 0.65% of lymphocytes were CD4⁺CD25⁺Foxp3⁺. After exposure to OVA aerosols (days 29/32), we observed a considerable increase in CD4⁺CD25⁺Foxp3⁺ cells (respectively, 1.45% and 1% of lymphocytes). A major difference appeared after INT (day 37), indeed the proportion of CD4⁺CD25⁺Foxp3⁺ T cells was remarkably higher in OVA INT mice than in PBS INT mice (1.4% vs. 0.5% of lymphocytes). This difference between OVA INT and PBS INT mice did not appear at later time-points (days 43/48/52). These results indicated that the INT with OVA resulted in an early induction of CD4⁺CD25⁺Foxp3⁺ T cells in the lungs. Subsequently, to assess suppressive activity *in vitro*, CD4⁺CD25⁺ T cells from the lungs of tolerized mice (OVA INT, day 52) and CD4⁺CD25⁻ T cells from the lungs of asthmatic mice (PBS INT, day 52) were co-cultured in the presence of OVA (Fig. 3B). CD4⁺CD25⁺ T cells suppressed the growth of CD4⁺CD25⁻ T cells in a dose-dependent manner. Furthermore, CD4⁺CD25⁺ T cells cultured alone were anergic with poor proliferation to OVA. Taken together, these *in vivo* and *in vitro* assays supported a regulatory phenotype for the CD25⁺ T cell fraction.

Ovalbumin intranasal treatment down-regulates airway eosinophilia despite short-term CD25⁺ T cells depletion

As CD25⁺ T cells depletion did abrogate induction of tolerance in asthmatic mice, we examined the effect of a short-term depletion limited to days 31–42 (80% depletion until day 42, data not shown) (Fig. 4A). At day 52, eosinophils absolute numbers in BALF of OVA INT/PC61 mice were comparable with OVA INT mice and considerably lower than PBS INT mice. As observed in the long-term depletion protocol, at day 52, T cell response to OVA in OVA INT/PC61 mice was significantly higher than in OVA INT mice and comparable with PBS INT mice. Globally, these data suggest that short-term depletion of CD4⁺CD25⁺ T cells did not affect low eosinophils recruit-

ment in lungs but was sufficient to reverse OVA specific T cell hyporesponsiveness.

Transfer of CD4⁺CD25⁻ T cells inhibits eosinophil recruitment into bronchoalveolar lavage fluid and leads to T cell hyporesponsiveness

CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells were isolated from lungs and BLN of tolerized (OVA INT) or non-tolerized mice (PBS INT) after challenge (day 52) and injected intravenously to asthmatic mice (day 35). Recipient mice were then challenged with OVA aerosols. Interestingly, only the transfer of CD4⁺CD25⁻ T cells from OVA INT mice and not from PBS INT mice significantly decreased both eosinophils recruitment into BALF of recipient mice (Fig. 5A) and T cell responses to OVA (Fig. 5B). However, both T cell subsets when isolated from OVA INT mice tended to decrease OVA-specific IgE in serum as compared with PBS INT or control mice (Fig. 5C). Cytokine (IL-5, IL-10) and eotaxin levels were measured in lung homogenates but were not modulated at these time-points by the transfer of any cell subset (data not shown). Our results suggest that more than CD4⁺CD25⁺ T cells, CD4⁺CD25⁻ T cells in our model were optimal regulators of lung inflammation when purified from OVA INT mice. Their transfer was indeed associated with three key immune events supporting induction of tolerance: down-regulation of eosinophil recruitment into BALF, T cell hyporesponsiveness and limitation of IgE increase upon re-exposure to allergen.

CD4⁺CD25⁻ T cells from tolerized mice express high levels of membrane-bound transforming growth factor- β

To characterize CD4⁺CD25⁺ and CD4⁺CD25⁻ T cell subsets *in vivo*, we first analyzed their Foxp3 expression. CD4⁺CD25⁺ T cells from BLN of donor OVA INT mice harvested at day 52 were, as expected, about 80% Foxp3⁺ whereas only 2–5% of CD4⁺CD25⁻ T cells expressed Foxp3 (Fig. 6A). Comparable results were obtained with donor PBS INT mice (data not shown). To evaluate the production of potentially anti-inflammatory cytokines at the cellular level, membrane-bound TGF- β (mTGF- β) expression was analyzed *ex vivo* at day 52 in CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells by flow cytometry. mTGF- β expression of lung CD4⁺ T cells was significantly higher in OVA INT mice than in PBS INT mice (data not shown, respectively, 1.1 $\pm$ 0.21% and 0.58 $\pm$ 0.04%). Remarkably, the frequency of mTGF- β ⁺ cells in CD4⁺CD25⁻ T cells was significantly higher in OVA INT mice than in PBS INT mice (8.35% vs. 4.57%) (Fig. 6B, left panel). In contrast, after overnight stimulation with OVA, the frequency of CD4⁺IL-10⁺ T cells remained low and comparable in OVA and PBS INT mice (data not shown, respectively, 0.39 $\pm$ 0.03% and 0.45 $\pm$ 0.04%). The frequency of IL10⁺ cells in CD4⁺CD25⁺

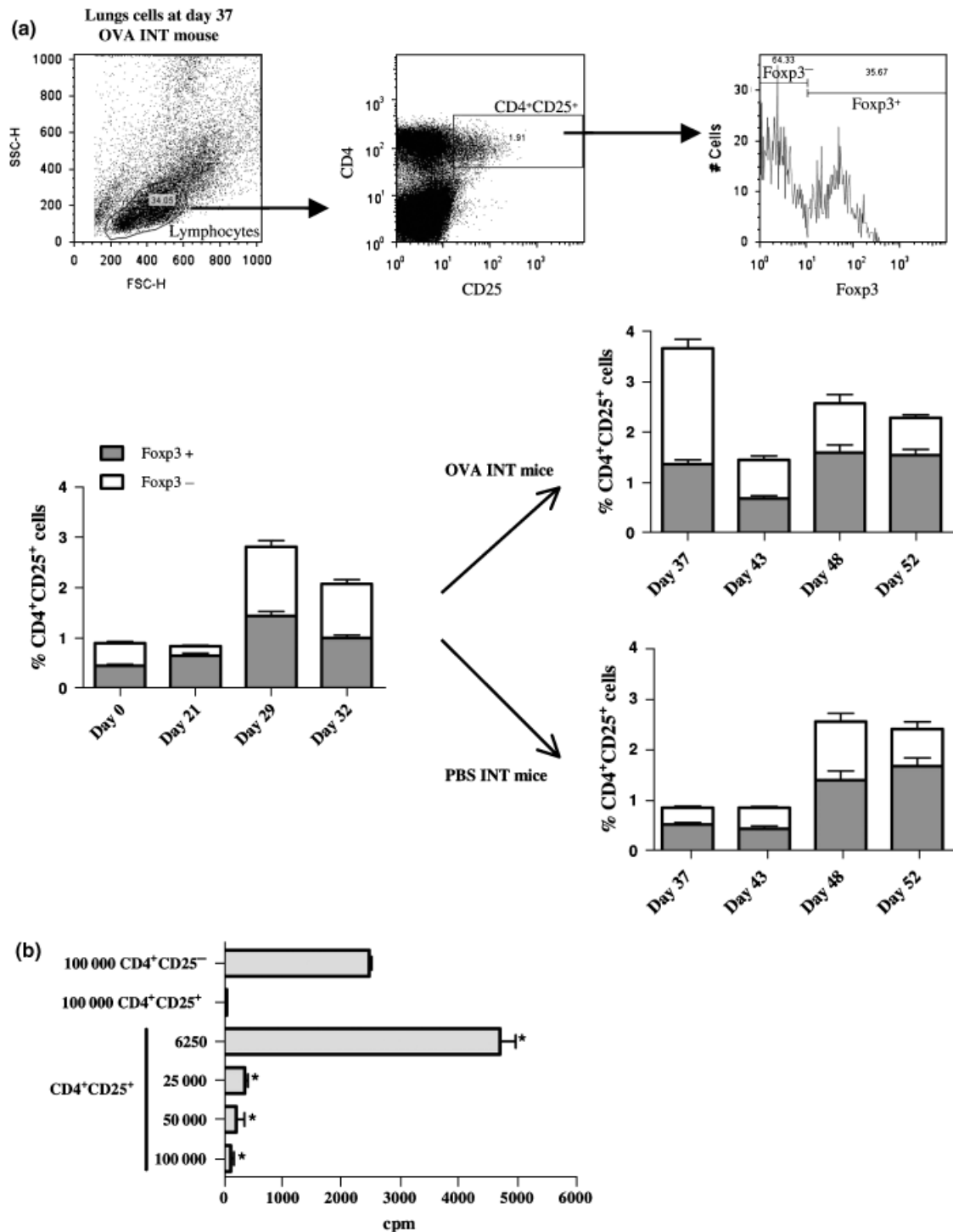


Fig. 3. CD4⁺CD25⁺Foxp3⁺ T cells are induced by OVA intranasal treatment and are suppressive *in vitro*. (a) Lungs cells from OVA INT and PBS INT mice were collected at days 0, 21, 29, 32, 37, 43, 48 and 52, stained with anti-CD4 mAb, anti-CD25 mAb, anti-Foxp3 mAb and analyzed by flow cytometry. Flow cytometry analysis for OVA INT mouse lungs at day 37 is described. Results are expressed as mean ± SD of 6–8 mice/group. (b) CD4⁺CD25⁺ T cells from tolerized mice (day 52) were cultured alone or with growing numbers of CD4⁺CD25⁺ T cells from asthmatic mice (day 52) in the presence OVA. [3H]-thymidine incorporation was measured after 3 days of culture. Results are expressed as mean cpm ± SD of duplicates from one representative experiment (*P < 0.05). INT, intranasal treatment; mAb, monoclonal antibodies; OVA, ovalbumin.

T cells was significantly higher in PBS INT mice (Fig. 6B, right panel), whereas the frequency of CD4⁺CD25⁺IL-10⁺ was similar in OVA and PBS INT mice. Most

CD4⁺CD25⁺mTGF-β⁺ and CD4⁺CD25⁺IL10⁺ T cells were Foxp3⁺ (>95%, data not shown) in this experiment and most probably corresponded to activated T cells.

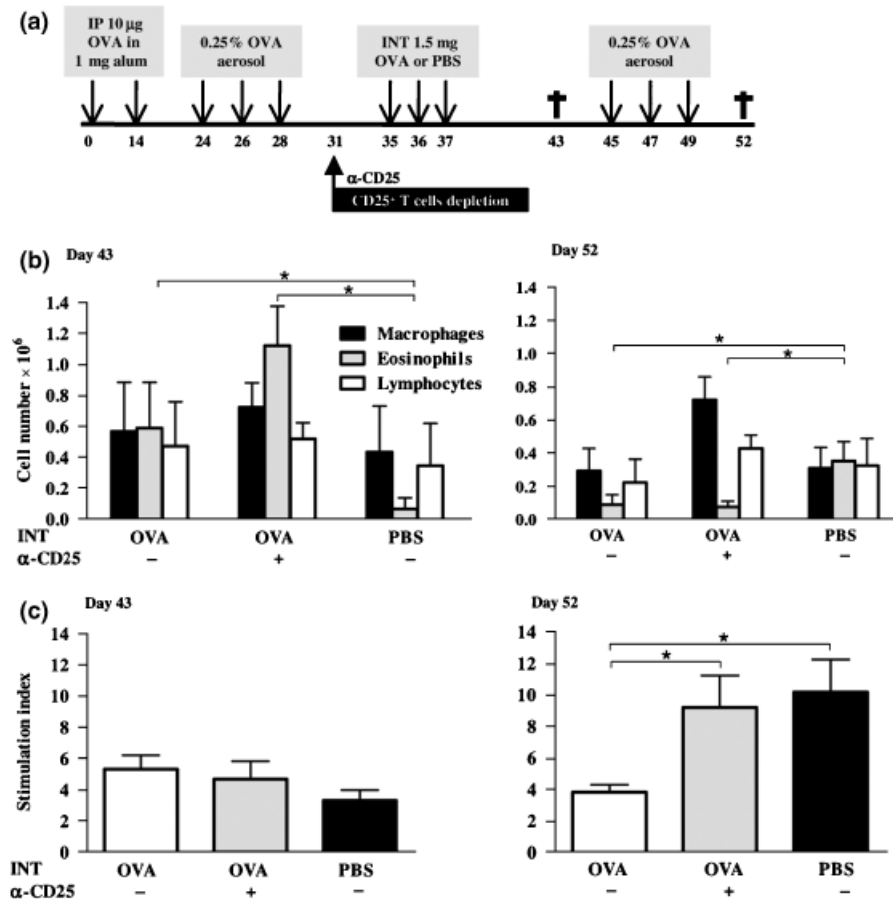


Fig. 4. Short-term depletion of $CD4^+CD25^+$ T cells abrogated T cell hyporesponsiveness. (a) Short-term depletion protocol: mice were treated as described in Fig. 1 but received only a single injection of 1 mg PC61 at day 31. (b) BALF eosinophils, macrophages and lymphocytes at day 52 (c) BLN cells were cultured for 4 days with OVA and proliferation was measured using [³H]-thymidine incorporation. Results are expressed as mean $\pm$ SD of 6 to 11 mice/group (* $P < 0.05$). BALF, bronchoalveolar lavage fluid; BLN, bronchial lymph nodes; OVA, ovalbumin.

Discussion

Regulatory T cells secreting anti-inflammatory cytokines IL-10 or TGF- β play a central role in the induction of tolerance in allergy [14, 16–20]. Nonetheless the respective role of $CD4^+CD25^+$ and $CD4^+CD25^-$ T regulatory cells in tolerance induction is still largely unexplored in the therapeutic models of established asthma. In this paper, using a model of induction of tolerance *via* the nasal mucosa, we showed that depletion of $CD4^+CD25^+$ T cells led to eosinophilic lung inflammation upon allergen challenge and abrogated antigen specific T cell hyporesponsiveness, in contrast to non-depleted mice. Furthermore, the transfer of $CD4^+CD25^-$ T cells from OVA-tolerized mice to asthmatic mice prevented optimally lung inflammation upon re-exposure to OVA. Taken together, these data strongly suggested that in this therapeutic model of airway inflammation both $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells played key role as suppressors of the Th2 inflammation *in vivo*. Moreover, we postulate that $CD4^+CD25^-$ T cells may need interactions with

$CD4^+CD25^+$ T cells to acquire regulatory properties. The precise modalities of these intercellular interactions will need to be further defined.

In OVA i.n. treated animals, $CD25^+$ T cells depletion resulted in enhanced recruitment of eosinophils to the lungs, enhanced IgG1/IgG2a ratio as well as enhanced antigen specific T cell proliferation. This strongly suggested that treatment with anti-CD25 mAb PC61 led to the depletion of cells with suppressive activity on the Th2 response. Indeed in this experimental setting, more than 95% $CD4^+CD25^+Foxp3^+$ T cells with regulatory activity were depleted. However, we must keep in mind that $CD25^+$ T cell depletion was non-specific because activated $CD4^+CD25^+$ T cells were also eliminated. Our data are in agreement with previous reports demonstrating that $CD4^+CD25^+$ T cells depletion before sensitization was associated with increased eosinophilic airways inflammation, IgE level and bronchial hyper-reactivity [10, 13, 21]. These reports were among the first ones to indicate a modulatory role of $CD25^+$ T cells on the suppression of the Th2 response in the lungs. Our data further extend

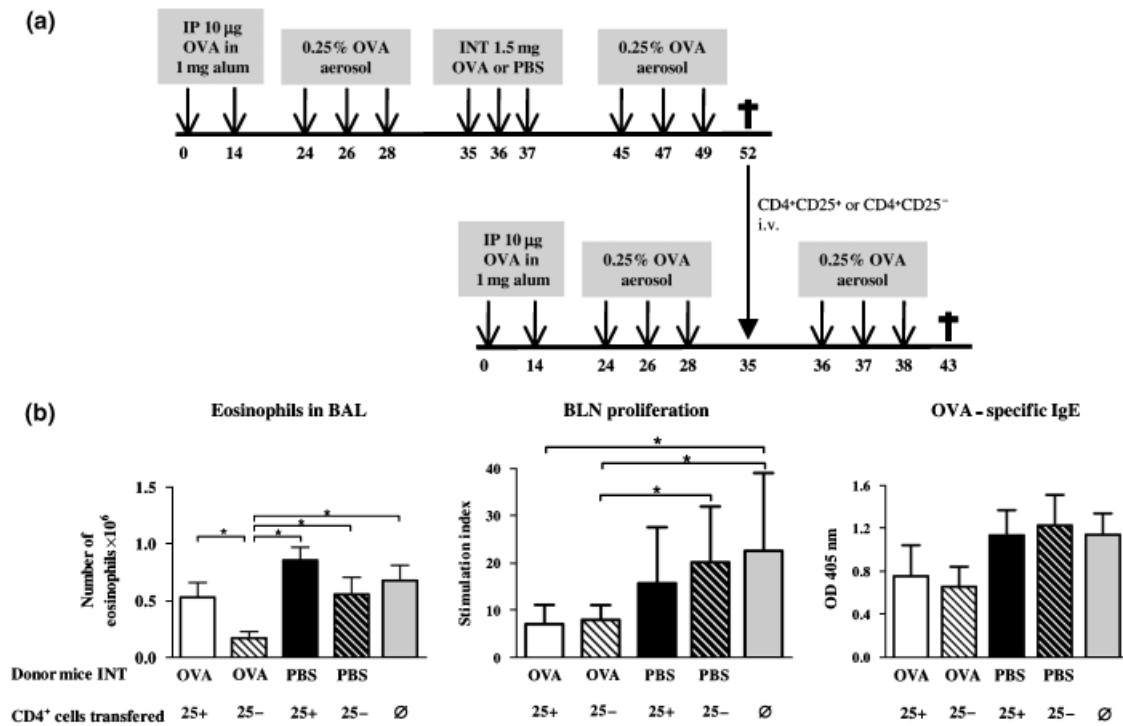


Fig. 5. Transfer of CD4⁺CD25⁻ T cells suppresses eosinophil recruitment into BALF and induce T cell hyporesponsiveness to OVA. (a) CD4⁺CD25⁻ cells from lungs and BLN of donor mice were collected at day 52 and transferred to recipient mice at day 35. Recipient mice were challenged with OVA aerosols at days 36/38/40. (b) At day 43: left panel, eosinophils in BALF; central panel, BLN cell proliferation to OVA; right panel, OVA specific IgE measured by indirect ELISA. Results are expressed as mean of 5 mice/group, one representative out of three experiments (**P* < 0.05). Groups of mice: Recipient mice that received CD4⁺CD25⁺ T cells from donor mice treated with OVA INT (OVA 25+), recipient mice that received CD4⁺CD25⁻ T cells from donor mice treated with OVA INT (OVA 25-), recipient mice that received CD4⁺CD25⁺ T cells from donor mice treated with PBS INT (PBS 25+), recipient mice that received CD4⁺CD25⁻ T cells from donor mice treated with PBS INT (PBS 25-), recipient mice that received only NaCl (∅). BALF, bronchoalveolar lavage fluid; BLN, bronchial lymph nodes; INT, intranasal treatment; OVA, ovalbumin.

these findings in demonstrating that down-regulation of the Th2 response by OVA INT was dependent on the presence of CD4⁺CD25⁺ T cells. Furthermore, tolerized OVA INT mice displayed increased level of serum OVA specific IgG1. This was in agreement with evidences demonstrating that efficient immunotherapy was associated with enhanced antigen-specific IgG1 responses [22]. CD25⁺ T cell depletion had no influence on this parameter.

To date, Foxp3 appears to be the most relevant marker for CD4⁺CD25⁺ regulatory T cells [23, 24]. In our model, CD4⁺CD25⁺ T cells were in a large majority Foxp3⁺ at day 52 in the lungs (68–79%) (Fig. 3A). As described in a large body of the literature [11, 25–27], CD4⁺CD25⁺ T cells from tolerized mice were anergic and suppressive *in vitro* (Fig. 3B). This suppressive activity was also reflected in proliferation assays *ex vivo*, because the T cell hyporesponsiveness observed in OVA INT mice was abrogated in OVA INT/PC61 mice at day 52 (Fig. 1C, right panel). T cell hyporesponsiveness in our model was a relatively late phenomenon (day 52). We know that, during the phase of tolerance induction, T cells undergo first a period of intense activation [15, 28]. Our observa-

tions are in agreement with this concept as BLN cells from OVA INT mice proliferated indeed more strongly than PBS INT mice at day 43 (6 days after INT) (Fig. 1C). Furthermore, at this time-point at least 50% of CD4⁺CD25⁺ T cells were Foxp3⁺-activated T cells (Fig. 3A). This situation was reversed at day 52 (21–32% Foxp3⁺ cells in CD4⁺CD25⁺ T cells), a date when T cell hyporesponsiveness was established in OVA INT mice.

When measuring global IL-10 and TGF-β levels in lungs homogenates, we did not identify significant differences between PC61 treated and untreated OVA INT animals (Fig. 2C). We cannot exclude that different sources of cytokines than T cells were implicated because dendritic cells secrete IL-10 and bronchial epithelial cells are a major source of TGF-β [12, 14]. Interestingly, there was a significant difference between OVA INT mice and OVA INT/PC61 mice in the production of IL-17 in the lungs. Higher levels of IL-17 in OVA INT mice may suggest a regulatory contribution of this cytokine in turning down the Th2 immune response of asthmatic mice in the absence of concomitant enhancement of the Th1 response [29]. We were indeed unable to detect any increase in IFNγ level in lung homogenates post-challenge with OVA

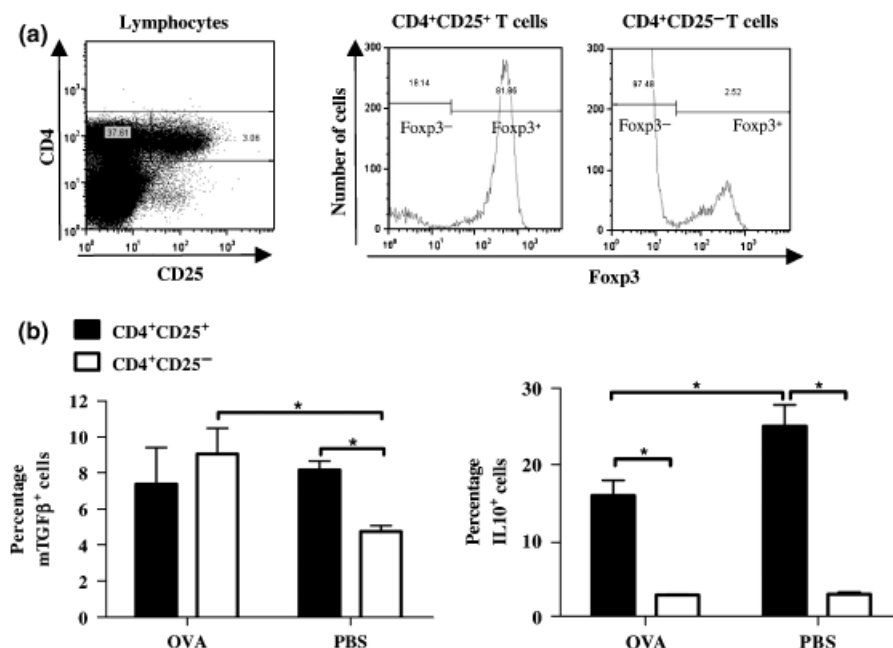


Fig. 6. Suppressive CD4⁺CD25⁻ T cells express a high level of membrane-bound TGF-β. (a) BLN cells from OVA INT were harvested at day 52, stained with anti-CD4 mAb, anti-CD25 mAb, anti-Foxp3 mAb and analyzed by flow cytometry. Results from a single representative mouse. (b) Frequency of mTGF-β⁺ and IL-10⁺ CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells from the lungs of OVA INT vs. PBS INT mice were analyzed by flow cytometry at day 52 after overnight stimulation with OVA. Results are expressed as mean of 5 mice/group, one representative experiment out of three (**P* < 0.05). BLN, Bronchial lymph nodes; INT, intranasal treatment; mAb, monoclonal antibodies; OVA, ovalbumin.

aerosols in CD25⁺ depleted as well as in non-depleted groups (data not shown). The induction of IL-17 in CD25⁺ non-depleted OVA INT mice appears in agreement with studies supporting an immunoregulatory activity of IL-17 in dampening the Th2 airways inflammation [30, 31]. Because the concomitant action of TGF-β and IL-6 is leading to the generation of Th17 cells [32], the IL-17 decrease associated with CD25⁺ cells depletion may result from the insufficient secretion/expression of TGF-β by CD25⁺ T cells. Furthermore, the stimulation of Th17 cells with TGF-β and IL-6 may also lead to the production of high amounts of the anti-inflammatory cytokine IL-10, and low amounts of inflammatory chemokines and IL-22 [29]. In the same model, inflammation induced by IL-23-stimulated Th17 cells was furthermore suppressed by IL-10 produced by Th17 cells expanded with TGF-β plus IL-6. This strongly supported a dual pro- and anti-inflammatory role for Th17 cells that will have to be further analyzed in this model of airway inflammation.

To better define the respective role of CD4⁺CD25⁺ T cells and CD4⁺CD25⁻ T cells in the down-regulation of airway inflammation, purified CD4⁺CD25⁺ and CD4⁺CD25⁻ T cell subpopulations were transferred to asthmatic animals. Both the transfer of CD4⁺CD25⁺ or CD4⁺CD25⁻ T cells from OVA INT mice (collected at day 52) but not from PBS mice was able to prevent lung inflammation (Fig. 5B). However, although the transfer of CD4⁺CD25⁻ or CD4⁺CD25⁺ T cells from OVA INT mice

led to antigen specific T cell hyporesponsiveness of recipient mice BLN cells, only the transfer of CD4⁺CD25⁻ T cells optimally and significantly inhibited eosinophil recruitment into BALF. Taking into account that long-term depletion of CD25⁺ T cells abrogated the induction of tolerance, we hypothesize that the capacity of CD4⁺CD25⁻ T cell subset to transfer tolerance was conditioned by the presence of CD25⁺ T cells in OVA INT mice. CD4⁺CD25⁺ T cells from OVA INT mice were suppressive *in vitro*, expressed Foxp3⁺ (>70%) and induced antigen specific T cell hyporesponsiveness *in vivo* when isolated from OVA INT mice but not from controls. These characteristics strongly suggested that these CD4⁺CD25⁺ Foxp3⁺ T cells were expressing features of inducible Treg. These data are in agreement with transfer experiments of CD25⁺ T cells from D011.10 OVA transgenic mice [8, 11], although we have not been able to show in our non-transgenic model a significant inhibitory effect of CD4⁺CD25⁺ T cells on BALF eosinophilia. About 30% of the CD4⁺CD25⁺ T cells were Foxp3⁻. We cannot exclude that these activated cells, or the transfer of an insufficient number of CD4⁺CD25⁺Foxp3⁺ OVA specific T cells may have contributed to the partial anti-inflammatory effect of CD4⁺CD25⁺ T cell subset. The model by itself, i.e. transfer of a transgenic T cell population from D011.10 mice vs. transfer of polyclonal non-transgenic T cells from BALB/c mice, may also play a role in this difference [8].

The potent anti-inflammatory activity of the CD4⁺CD25⁺ T cell subset is reminiscent of several previous reports, where in contrast to CD25⁺ T cells, the *in vivo* transfer of CD25⁺ T cells was able to induce protection [33–36]. It is unlikely that <5% CD4⁺CD25⁺Foxp3⁺ T cells contaminating our transferred preparation may account for this effect because >70% pure CD4⁺CD25⁺Foxp3⁺ population was not able to significantly decrease BALF eosinophilia (Fig. 5A). Interestingly, short-term depletion of CD4⁺CD25⁺ T cells in OVA INT mice during a limited period of time (Fig. 4A) did not impair the beneficial effect of OVA INT on airway eosinophilia. We postulate here a role for antigen presenting cells, most probably dendritic cells [37] that, following OVA sampling during INT, maintained their capacity to later interact with CD4⁺CD25⁺ T cells once regenerated, i.e. about 7–10 days after the first nasal application of OVA. This further supports the hypothesis that the progressive full regeneration of the CD4⁺CD25⁺ T cell pool from days 42–45 was crucial for the induction of CD4⁺CD25⁺ T cells with regulatory properties, able to protect airways upon OVA challenge. Interestingly, if CD25⁺ transiently depleted mice, like OVA INT mice, displayed limited number of eosinophils in BALF (Fig. 4B), T cells from BLN still exhibited a strong proliferative capacity at day 52 (Fig. 4C). This suggested differences in the immune regulation controlling eosinophil recruitment vs. T cell proliferation.

To approach the regulatory mechanisms of CD4⁺CD25⁺ T cells, we examined their expression of mTGF- β , another key player of tolerance induction in the airways [38–40]. Whereas, the frequency of CD4⁺CD25⁺mTGF- β ⁺ T cells were comparable in OVA INT vs. PBS INT mice, this frequency was significantly enhanced in the CD4⁺CD25⁺ T cell subset from OVA INT mice. This opens the question of the anti-inflammatory mechanisms developed by this cell subset that will have to be further examined. The role of IL-10 is debatable in our model because the frequencies of CD4⁺CD25⁺IL-10⁺ T cells were low and comparable in tolerized and non-tolerized mice. Furthermore, most mTGF- β ⁺ and IL-10⁺ CD25⁺ T cells were Foxp3 negative, suggesting that these T cells were most probably activated T cells. In this context, the greater proportion of CD4⁺CD25⁺IL-10⁺ T cells in asthmatic animals compared with tolerized mice supported a pro-inflammatory role of IL-10⁺ T cells. With regards to the enhanced levels of IL-10 measured in lung homogenates of OVA INT mice (Fig. 2C), we may have to consider other sources for IL-10 than T cells, for instance dendritic cells, although we cannot exclude that despite a similar frequency, the production of IL-10 by CD4⁺CD25⁺ may be enhanced in OVA INT mice as compared with CD4⁺CD25⁺ T cells. These aspects will have to be further analyzed.

Taken together, our data indicate that, in OVA sensitized mice, during a brief time frame post OVA nasal adminis-

tration (from days 37–42), CD4⁺CD25⁺ T cells contributed to the down-regulation of the allergic response *via* the generation of CD4⁺CD25⁺ T cells with regulatory properties. The relationships and interactions between the CD4⁺CD25⁺ and CD4⁺CD25⁺ T cell subsets will have to be precisely defined. The respective mechanisms of action of CD4⁺CD25⁺ and CD4⁺CD25⁺ regulatory T cells involved in this model of tolerance induction by INT with OVA will have to be further explored as well, i.e. direct cell-to-cell contact vs. anti-inflammatory cytokine secretion, with a potentially important role for Th17 cells.

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