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$CD4^+CD25^-mTGF\beta^+$ T cells induced by nasal application of ovalbumin transfer tolerance in a therapeutic model of asthma

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Abstract

Background: **Intranasal administration of high amount of allergen was shown to induce tolerance and to reverse the allergic phenotype. However, mechanisms of tolerance induction via the mucosal route are still unclear.** ***Objectives:*** **To characterize the therapeutic effects of intranasal application of ovalbumin (OVA) in a mouse model of bronchial inflammation as well as the cellular and molecular mechanisms leading to protection upon re-exposure to allergen.** ***Methods:*** **After induction of bronchial inflammation, mice were treated intranasally with OVA and re-exposed to OVA aerosols 10 days later. Bronchoalveolar lavage fluid (BALF), T cell proliferation and cytokine secretion were examined. The respective role of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells in the induction of tolerance was analysed.** ***Results:*** **Intranasal treatment with OVA drastically reduced inflammatory cell recruitment into BALF and bronchial hyperresponsiveness upon re-exposure to allergen. Both OVA- specific-proliferation of T cells, T_h1 and T_h2 cytokine production from lung and bronchial lymph nodes were inhibited. Transfer of $CD4^+CD25^-$ T cells, which strongly expressed membrane-bound transforming growth factor β (mTGFβ), from tolerized mice protected asthmatic recipient mice from subsequent aerosol challenges. The presence of $CD4^+CD25^+(Foxp3^+)$ T cells during the process of tolerization was indispensable to $CD4^+CD25^-$ T cells to acquire regulatory properties. Whereas the presence of IL-10 appeared dispensable in this model, the suppression of $CD4^+CD25^-mTGF\beta^+$ T cells in transfer experiments significantly impaired the down-regulation of airways inflammation.** ***Conclusion:*** **Nasal application of OVA in established asthma led to the induction of $CD4^+CD25^-mTGF\beta^+$ T cells with regulatory properties, able to confer protection upon allergen re-exposure.**

Keywords: asthma, CD25, immunotherapy, TGFβ, tolerance, T regulatory cells

Introduction

Although T_h2 polarization cannot fully explain a complex allergic condition such as asthma (1), allergen-specific immunotherapy (SIT) in human was shown to derive the allergen-specific T_h2 immune response towards a T_h1 response (2–4) and in parallel to enhance a T regulatory cell response based on activation of IL-10-producing $CD4^+CD25^+$ T cells (5–7). In addition, several studies demonstrated the efficacy of SIT not only in improving asthma symptoms (8, 9) but also in delaying the progression from allergic rhinitis to asthma in children (10). Hence, induction of tolerance to allergen appears as an efficient treatment strategy to establish both an early prophylactic as well as a therapeutic control of the specific mechanisms leading to allergic inflammation.

In naive animals, application of high-dose allergen on the respiratory or nasal mucosa was able to hamper the development of airway hyperresponsiveness (AHR) and/or T_h2-polarized responses (11–13). Two cell lineages were considered key players in the induction of immune tolerance to allergen: dendritic cells (DCs) and $CD4^+$ T cells (14). In prophylactic models, the development of respiratory tolerance was initiated by uptake of antigen in the lungs by immature DC that continuously sampled foreign antigens in

the bronchial mucosa (15). As proposed in early studies, after exposure to ovalbumin (OVA), immature pulmonary DC migrate to the draining bronchial lymph nodes (BLN) where they mature and initiate a phase of allergen-specific T-cell activation, proliferation and expansion, followed by T-cell depletion from lymphoid organs. A stable population of T cells could survive but remained refractory to antigenic re-exposure (anergy) (12, 14, 16).

Whereas nasal application of allergen or allergen-derived peptides significantly improved established systemic hypersensitivity to OVA or phospholipase A_2 (16, 17), the possibility of reducing bronchial inflammation in established asthma via the nasal route is still debated (13, 18). Mechanisms of protection are yet incompletely understood although a role for IL-10, transforming growth factor (TGF) β and $CD25^+$ T cells has been suggested (19–23). Furthermore, we have recently shown that $CD4^+CD25^+(Foxp3^+)$ T-cell depletion fully abrogated tolerance induction via the intranasal route in an experimental model of asthma (24). Therefore, to evaluate the therapeutic potential of antigen SIT via the nasal route and to better understand the mechanisms of induction of tolerance to allergen, we further dissected our experimental mouse model of asthma to OVA that displays full blown features of reversible airway inflammation and provided conditions compatible with allergen seasonal exposure in human.

Methods

Induction of asthma and intranasal treatment

Six- to 8-week-old BALB/c mice (Harlan, Horst, The Netherlands) were sensitized twice at day 0 (D0) and D14 by intraperitoneal (i.p.) injections of 10 μg OVA (LPS <15 pg mg^{-1}; Fluka, Buchs, Switzerland) adsorbed on 1 mg aluminum hydroxide (Sigma Chemicals, St Louis, MO, USA). At D24, D26 and D28, animals were exposed to a single aerosol of OVA in PBS (0.25%) (Fig. 1 window). Aerosolization was generated by a nebulizer (De Vilbiss; Sunrise medical GmBH, Malsch, Germany) for 20 min. At D38, 39 and 40 mice were treated intranasally with OVA 0.5 mg, 1.0 mg or 1.5 mg [OVA intranasal treatment (INT) group] or PBS (control group; PBS

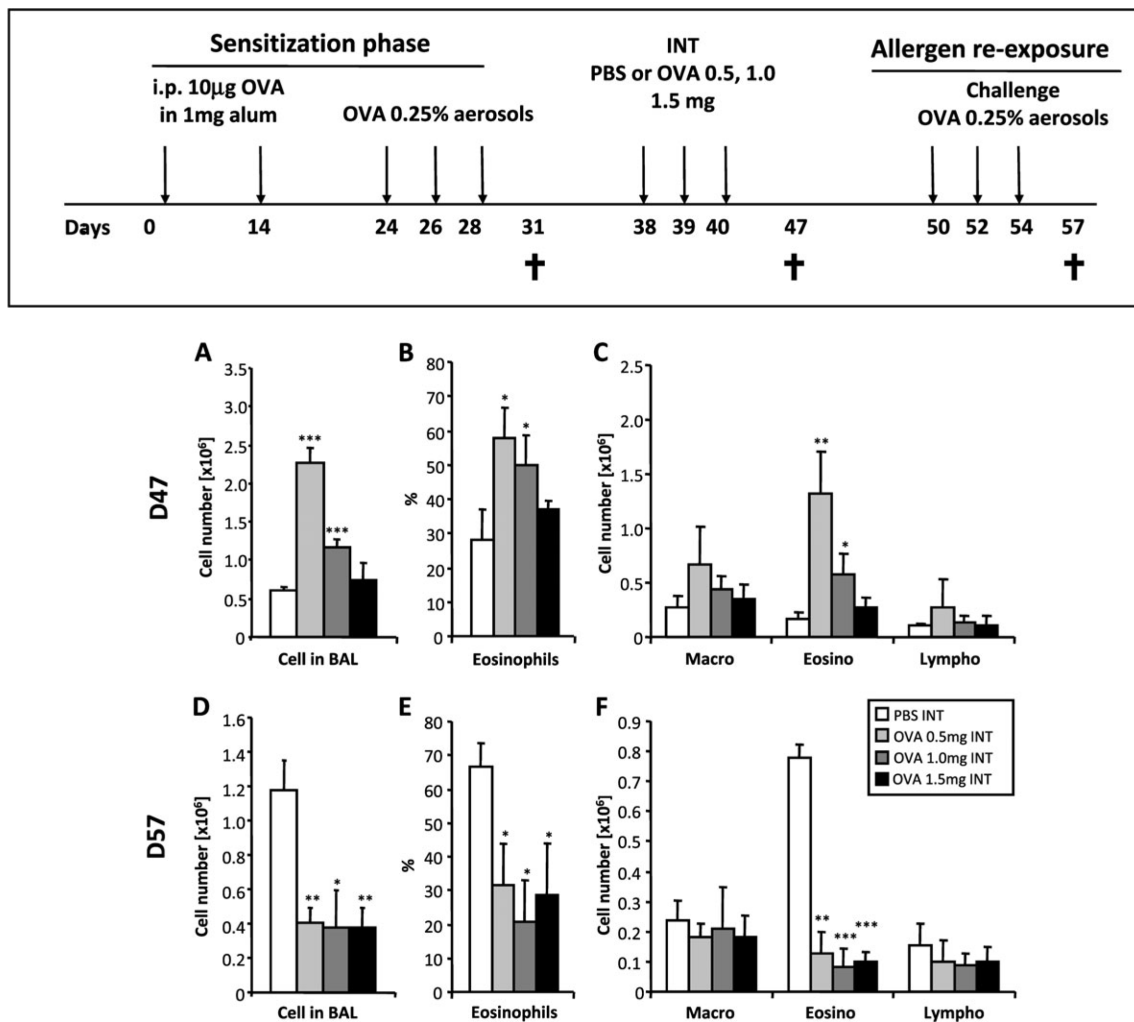

Fig. 1. INT with OVA protects against cell recruitment into BALF upon OVA aerosol challenge. Total BALF cell number (A and D), relative eosinophil count in % (B and E) and mononuclear cell subpopulations number (C and F) in BALF harvested after INT (D47) or after challenge (D57), in animals treated intranasally with 0.5, 1.0 or 1.5 mg OVA versus PBS. *P < 0.05; **P < 0.01; ***P < 0.001. Window: model's treatment scheme; crosses indicate sacrifice.

INT group) or human serum albumin (0.25%; CLS Behring, Switzerland) when indicated (Fig. 2C and D), under light anaesthesia with halotane (Halocarbon B.P.; Arovet A.G., Zollikon, Switzerland). Mice were finally challenged with 0.25% OVA aerosol at D50, D52 and D54 and sacrificed at D57. When indicated (Fig. 8), wild-type C57BL/10 and C57BL/10 $IL10^{-/-}$ mice (B10.129P2(B6)-$Il10^{tm1Cgn}$/J) (Charles River, France) maintained in pathogen-free environment were treated following the same protocol.

Bronchoalveolar lavage and AHR

Bronchoalveolar lavage was performed by injecting a total of 3 ml PBS (6 × 500 μl) into the lung. Approximately 10^5 cells in 100 μl were centrifuged on glass plates (Cytospin; Shandon scientific, Cheshire, U.K) and then stained with DiffQuik according to the manufacturer's recommendations (Baxter Dade, Dudingen, Switzerland). A differential count of 200 cells was performed using standard morphological criteria. AHR was measured in conscious unrestrained mice using barometric whole-body plethysmography (Buxco; EMKA technologies, Paris, France). Respiratory pressure curves were recorded in response to inhaled methacholine (Sigma Chemicals).

Proliferation assay

Proliferation assays were done as previously described (16). Briefly, 1 × 10^5 BLN cells or 5 × 10^5 splenocytes were stimulated with or without 5 μg ml^{-1} OVA for 4 days, and cell proliferation was measured by ^{3}H-thymidine incorporation. Alternatively, $CD4^+$-enriched T cells (using magnetic beads from BD PharMingen, San Diego, CA, USA) from spleens of DO11.10 mice were labelled with carboxyfluorescein succinimidyl ester (CFSE) (Molecular probes, Invitrogen, Carlsbad, CA, USA) (25). At D54, 3 × 10^6 KJ1-26$^+$$CD4^+$ T cells were injected into the tail vein of asthmatic BALB/c mice following previous protocols (26). Recipient mice were sacrificed at

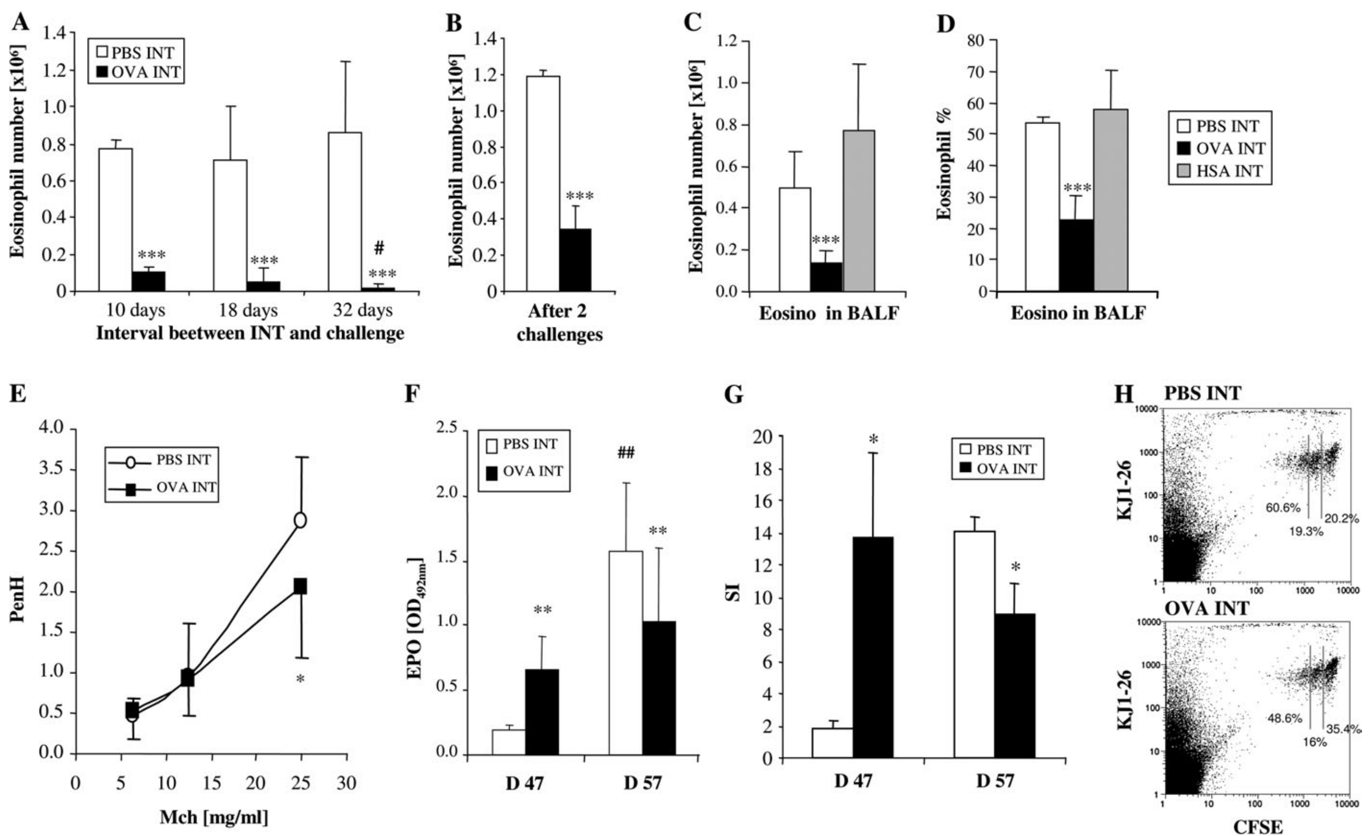

Fig. 2. OVA INT protects against re-exposure to OVA aerosol for an extended period of time in an antigen-specific manner and limits bronchial hyperresponsiveness and T-cell proliferation. (A) Eosinophil number in BALF after extension of interval between INT and challenge to 10, 18 or 32 days. *compared with PBS; #compared with 10 days. (B) Sensitized mice were challenged twice 30 and 60 days after INT, and eosinophils were quantified in BALF 3 days after the last challenge. (C and D) Induction of tolerance is antigen specific: INT was done either with PBS, 1.5 mg OVA or 1.5 mg human serum albumin in OVA-sensitized mice. Eosinophil number (C) and eosinophil percentage (D) in BALF at D57. (E) At D56, PenH was evaluated by whole-body plethysmography after stimulation with indicated methacholine concentrations. (F) Total lung EPO activity at D47 and D57, expressed as optical density (OD). *compared with PBS; #compared with D47. (G) BLN and spleen cells proliferation in response to 5 μg ml^{-1} OVA. Data are expressed as stimulation indexes (SI). (H) CFSE-labelled-$CD4^+$-enriched splenocytes from DO11.10 mice were transferred in OVA-sensitized BALB/c mice intranasally treated with OVA (lower panel) or PBS as a control (upper panel). Mice were sacrificed at D57 and single-cell suspension of BLN were labelled with surface antibodies against KJ1-26 (DO11.10 specific) (BD PharMingen). Samples were analysed on a FACScalibur® and fluorescence was determined using CellQuest® software (BD Biosciences). Numbers indicate percentage of cells undergoing more than one, one or no division. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

D57 (3 days post-aerosol challenges). CFSE-labelled KJ1-26$^+$ T-cell divisions were detected by flow cytometry.

Antibody isotypes

Serum IgE, IgG1 and IgG2a antibody response was determined by ELISA as described (16, 27). Alternatively, IgE were measured by indirect ELISA. Briefly, plates coated with 2 μg ml^{-1} rat anti-mouse IgE mAb (BD PharMingen) were incubated with mouse serum (1:100) for 2 h at 37°C, then with 500 ng ml^{-1} biotinylated OVA and revealed with alkaline phosphatase.

Cell preparation and inflammatory markers measurement

Lungs were harvested, snap frozen in dry ice and kept at −80°C until use. Frozen lungs were homogenized in a Dounce tissue grinder with 1 ml PBS containing protease inhibitors (Complete, Roche Diagnostics GmbH, Basel, Switzerland) and centrifuged for 4 min at 1640 × g. Supernatants of lung homogenates were collected for cytokines measurements. In addition, cytokines were measured in supernatants from BLN or lung cells cultured for 3 days at 37°C in 5% CO_2. A total of 10^6 fresh cells from BLN or 3×10^6 fresh cells from the lungs were seeded in 48- (BLN) or 24- (lung) wells plates with 125 μg ml^{-1} OVA in DMEMc. (DMEM (Gibco-BRL, Life technologies, AG, Basel, Switzerland) supplemented with 50 mM beta-mercaptoethanol (Fluka, Buchs, Switzerland), 2mM L-glutamine, 1mM sodium-pyruvate, 1mM HEPES, 100 IU/mL penicillin-streptomycin and 10% FCS (Gibco-BRL)). Cytokines levels were determined by ELISA according to the manufacturers' recommendations (BD PharMingen for IL-5 and IFNγ; R&D Systems, Minneapolis, MN, USA, for IL-10, IL-4, IL-13 and TGFβ). Eosinophil peroxidase (EPO) activity was measured in lung homogenates processed as described in ref. (28) and according to Strath *et al.* (29).

Cell enrichment and transfer

Lungs from PBS INT or OVA INT mice were harvested at D52; single-cell suspension were performed and thereafter enriched in CD4$^+$ T cells on magnetic beads according to the manufacturer (BD Biosciences) (Fig. 7A). More than 90% CD4$^+$ pure fractions were obtained. Where indicated, CD25$^+$ cells or CD25$^+$mTGFβ$^+$ cells were positively selected by magnetic cell sorting according to the manufacturer (Miltenyi Biotech GmBH, Bergisch Gladbach, Germany). Purity was typically >85%. A total of 1×10^6CD4$^+$ or 5×10^5CD4$^+$CD25$^+$ or CD4$^+$CD25$^-$ cells were re-suspended in NaCl 0.9% and 100 μl were injected in the tail vein of OVA-sensitized mice at D35. Recipient mice were challenged with OVA aerosol 1, 3 and 5 days after cell transfer and sacrificed 3 days later (D43).

Anti-CD25 mAb 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. Mice were treated i.p. with 1 mg PC61 at days 31, 38 and 45 (Fig. 7A). A single i.p. treatment with 1 mg PC61 yielded >80% CD4$^+$CD25$^+$ T cells depletion in PBMC and spleen during 7 days (24).

Flow cytometry

IL-10 expression was monitored using the 'MACS Mouse IL-10 secretion assay kit' (Miltenyi Biotech). According to the manufacturer, single-cell suspensions from BLN or lungs stimulated for 3 days with OVA (125 μg ml^{-1}) were labelled with antibodies to IL-10, CD4 and CD45R/B220 (BD PharMingen). IL-10-secreting cells were further labelled with anti-PE magnetic microbeads and enriched over a MACS separator system. Membrane-bound transforming growth factor β (mTGFβ) expression on BLN cells was performed with an anti-TGFβ mAb (IQ Products, Groningen, The Netherlands). Samples were analysed on a FACScalibur® and fluorescence intensity was determined using CellQuest® software (both from BD Biosciences).

Statistical analysis

Results are expressed as mean ± SD of four to six mice per group. Each experiment was done at least three times. Significance of differences between means was determined by Student's *t*-test using Prism 4.00 software for Windows (GraphPad Software Inc., La Jolla, CA, USA).

Results

INT with OVA inhibits inflammatory cell recruitment into bronchoalveolar lavage fluid (BALF) upon re-exposure to OVA.

To induce bronchial inflammation, mice were first sensitized by two i.p. injections of OVA and a series of three OVA aerosols as described in Methods. At day 31, these mice displayed a strong eosinophil recruitment (79.07 ± 1.99%, $1.66 \pm 0.32 \times 10^6$ eosinophils in the BALF as compared with control PBS-aerosolized mice: 3.12 ± 0.42%; $0.03 \pm 0.003 \times 10^6$). OVA-aerosolized mice were then randomly assigned to PBS INT or OVA INT group at day 38 and treated intranasally with OVA in a dose-escalating schedule (0.5, 1.0 and 1.5 mg) every day for 3 days from D38. Control mice received PBS alone. One week after INT with OVA (D47), an enhanced inflammatory cell recruitment (total cell and eosinophil count) was observed in BALF, which was inversely correlated to the intranasal dose of OVA (Fig. 1A–C). On the contrary, after challenge with OVA aerosols (D57), inflammatory cell recruitment was markedly impaired, however, without evidence of a dose effect (Fig. 1D–F). These data clearly indicated that INT with OVA was able to protect allergen re-exposed mice from recruiting eosinophils and, to a lesser extent, lymphocytes into the bronchial lumen, both major players of airway inflammation. Inhibition of eosinophil recruitment at D57 was preceded by apparent activation of inflammation post-INT in an inversely correlated dose response at D47.

To evaluate the duration of protection, the interval between INT with OVA and re-exposure to OVA aerosols was extended from 10 to 18 or 32 days. Longer time intervals further attenuated BALF eosinophil recruitment (Fig. 2A). Moreover, when aerosol challenges with OVA were repeated twice at 30-day interval, eosinophil recruitment appeared to be durably and strongly inhibited (Fig. 2B). Inhibition of eosinophil recruitment into BALF was antigen specific since INT with human albumin (HAS) in OVA-sensitized mice had no effect on eosinophil recruitment into BALF upon OVA aerosol challenge (Fig. 2C and D).

OVA INT inhibits AHR, EPO production and cell proliferation

To determine whether inhibition of eosinophil influx into BALF was also accompanied by a decrease in other parameters of lung inflammation, AHR, EPO and lymphocytes proliferation were assessed after re-exposure to OVA aerosols (D57). At this time point, AHR and EPO activity significantly decreased in OVA INT mice (Fig. 2E and F), whereas significantly higher levels of EPO were detected at D47 (Fig. 2F). These data suggested that INT with OVA not only inhibited eosinophil recruitment into bronchial lumen but also in lung tissue itself. Finally, T-cell proliferation assays to OVA led to marked allergen-specific T-cell hyporesponsiveness at D57 (Fig. 2G). To further support this observation, CFSE-labelled OVA transgenic T cells from D011.10 mice were transferred into OVA INT mice at D54. Tracked by cytofluorimetry at D57, they divided less actively as compared with PBS INT mice (Fig. 2H).

OVA INT inhibits IgE production upon allergen re-exposure and generates competitive anti-OVA IgG

After OVA challenge (D57), a strong production of OVA-specific IgE was induced in control mice, whereas IgE in OVA INT mice remained at baseline level in contrast to OVA-specific IgG1 (Fig. 3A and B). IgG2a production was similar in OVA- and PBS-treated mice (Fig. 3C). To evaluate a potential competition between OVA-specific IgG and IgE for binding sites, an anti-IgE capture ELISA was performed as an alternative approach. In this condition, we observed a strong IgE production in the OVA INT group at D47, which remained constant after challenge (D57) (Fig. 3D), indicating that the low IgE levels observed after challenge in OVA INT mice resulted both from inhibition of IgE production and competition with specific anti-OVA IgG.

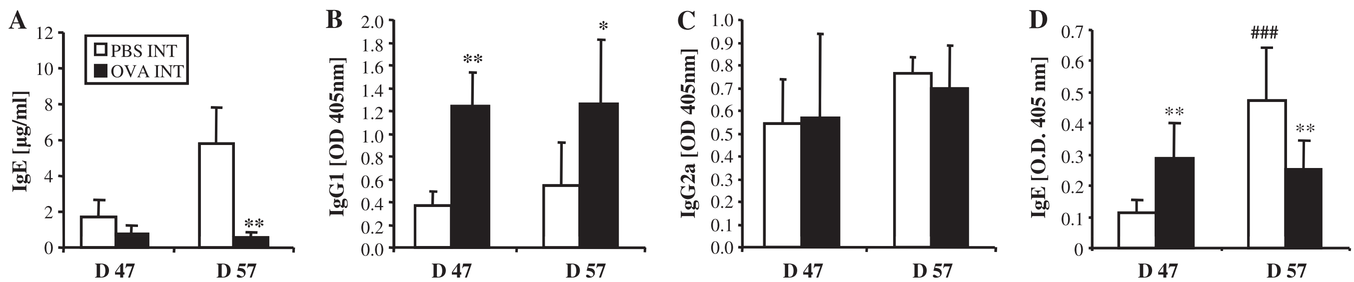

Fig. 3. Regulation of OVA-specific IgE by OVA INT (1.5 mg). Sera were collected at D47 and D57. OVA-specific IgE (A), IgG1 (B) and IgG2a (C) were quantified by ELISA. (D) OVA-specific IgE quantified by indirect method to avoid interactions with IgG. Dilutions of mouse serum were 1:20 for IgE, 1:500 000 for IgG1 and 1:1000 for IgG2a. *compared with PBS; #compared with D47. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

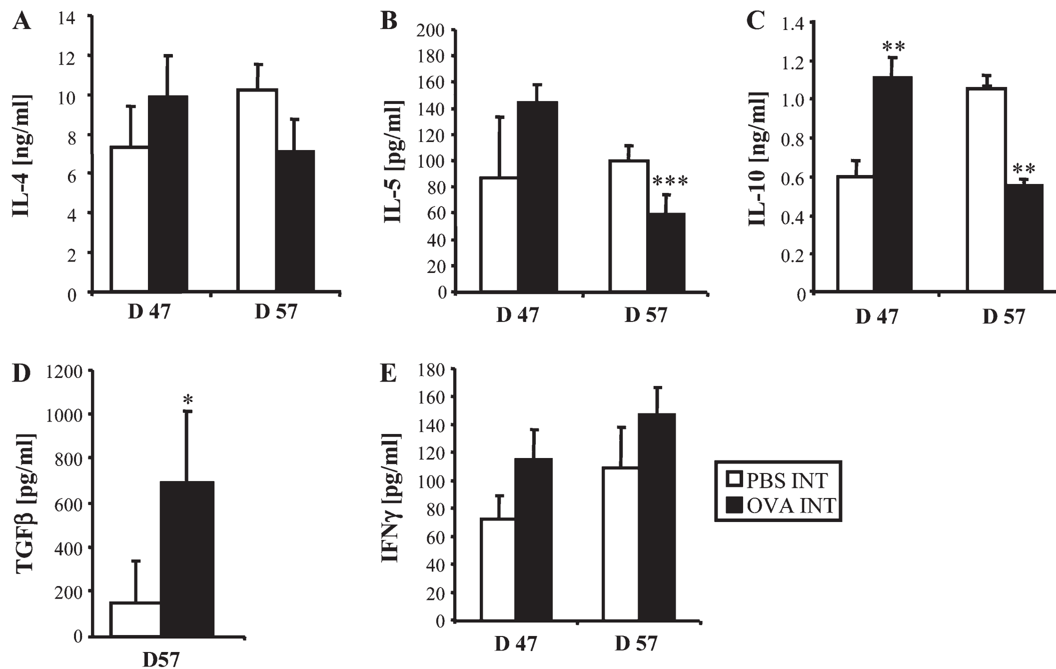

Fig. 4. OVA INT (1.5 mg) prevents cytokines production in lungs upon allergen challenge. Lungs were harvested at D47 (after INT) and D57 (after challenge). Cytokines were measured by ELISA in supernatants of lung homogenates. (A) IL-4, (B) IL-5, (C) IL-10, (D) TGFbeta, (E) IFNγ. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

OVA INT prevents T_h2 cytokine production upon re-exposure to OVA

In supernatants of lung homogenates, IL-4, IL-5 and IL-10 production followed the same kinetics: a vigorous induction after INT (D47) and a marked inhibition upon re-exposure to OVA in OVA INT mice (D57) (Fig. 4A–C), in contrast to TGFβ strongly stimulated after OVA re-exposure (Fig. 4D). IFNγ production did not significantly differ between groups at D47 and D57 (Fig. 4E). In the supernatants of BLN or lung cells stimulated with OVA, IL-4, IL-5, IL-10, IL-13 and IFNγ, production did not significantly differ at D47 between OVA INT mice and controls (Fig. 5A–E), except for IL-10, which significantly increased in BLN cells of OVA INT mice (Fig. 5C). In contrast, 3 days after re-exposure to OVA, BLN and lung cells of OVA INT mice produced significantly less IL-4, IL-5, IL-10 and IL-13 than cells from controls. Production of IFNγ by BLN cells of OVA INT mice was also significantly lower than in control animals. At this time point, IFNγ was repetitively undetectable in lung cells. To further investigate the potential role of IL-10 in the down-regulation of the immune response, the frequency of $CD4^+$ IL-10^+ T cells was also measured by flow cytometry. Following a profile similar to IL-10 production in T-cell supernatants, INT with OVA was associated with an enhanced frequency of $CD4^+$ IL-10^+ T cells at D47, whereas at D57 $CD4^+$ IL-10^+ T cells expansion was markedly limited (Fig. 5F).

$CD4^+CD25^-$ T cells protect recipient mice against OVA challenge

To identify the key regulatory cell subsets, we first performed a preliminary transfer experiment with total $CD4^+$ T cells from BLN and lung from OVA INT mice into OVA-sensitized recipients. Transfer efficiently inhibited eosinophil recruitment into the BALF of recipient asthmatic mice at D57 (Fig. 6A and B). OVA-specific serum IgE as well as IL-4 and IL-10 in lung supernatants were also down-regulated (Fig. 6C–E). We next transferred $CD4^+CD25^+$ and $CD4^+CD25^-$ cells from OVA INT or PBS INT mice to OVA-sensitized mice. $CD4^+CD25^-$ T cells from OVA INT, but not from PBS INT, mice protected recipient mice from recruiting eosinophil into BALF upon allergen re-exposure. The transfer of $CD4^+CD25^+$ T cells only partially suppressed BALF eosinophilia (Fig. 6F). As measured by flow cytometry, the frequency of $CD4^+CD25^-mTGF\beta^+$ T cells was significantly enhanced in OVA INT mice at D57 (Fig. 6G) in contrast to that of $CD4^+CD25^+mTGF\beta^+$ T cells that did not differ between groups (data not shown).

$CD4^+CD25^-mTGF\beta^+$ T cells play a key role in the induction of tolerance

As suggested above (Fig. 6G), $mTGF\beta^+$ subset of $CD4^+CD25^-$ T cells may play a role in the induction of tolerance upon INT with OVA in our model. To evaluate the role of mTGFβ-expressing $CD4^+CD25^-$ T cells, $CD4^+CD25^-$ T cells were first purified from animals treated intranasally with OVA then depleted by magnetic cell sorting from mTGFβ-expressing $CD4^+CD25^-$ T cells. BALF from OVA-sensitized/OVA aerosol-exposed animals transferred with mTGFβ-expressing $CD4^+CD25^-$ T cells were significantly less

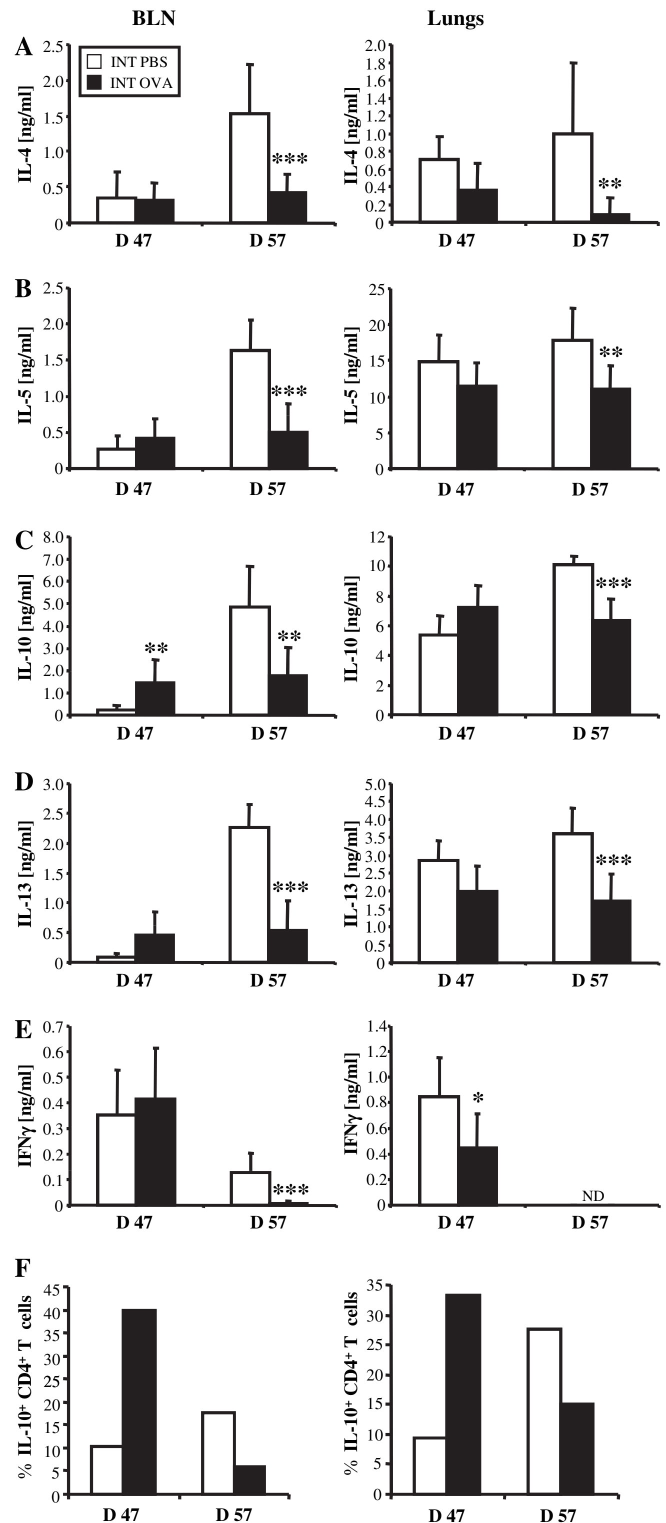

Fig. 5. OVA INT (1.5 mg) prevents cytokines production by BLN and lung cells 3 days after allergen challenge. BLN and lungs were harvested at D47 and at D57. IL-4 (A), IL-5 (B), IL-10 (C), IL-13 (D) and IFNγ (E) were measured in culture supernatants by ELISA. Results are expressed as mean ± SD of eight mice per group. ND, not detectable. (F) Flow cytometric analysis of $CD4^+$ IL-10^+ T cells from a pool of BLN (left panel) or lung (right panel) cells isolated from five mice. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

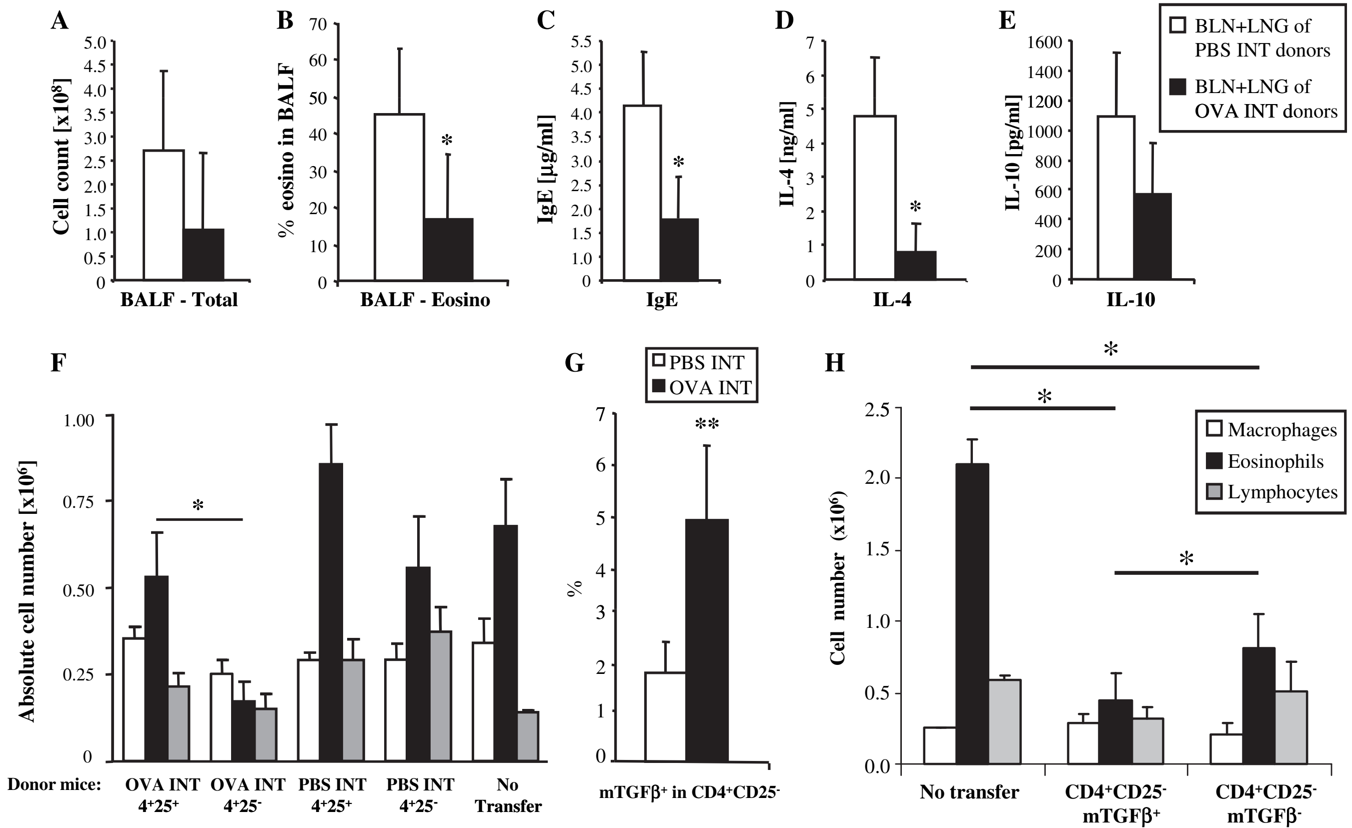

Fig. 6. Transfer of $CD4^+CD25^-$ cells from OVA INT mice protects against OVA challenge. (A–E) A total of 1×10^6 $CD4^+$ cells of OVA INT (1.5 mg) or PBS INT mice harvested at D52 were suspended in 100 µl saline 0.9% and injected in the tail vein of recipient OVA-sensitized mice at D35. Aerosol challenge was performed 1, 3 and 5 days after transfer and mice were sacrificed 3 days later. (A) Total cells in BALF of recipient mice. (B) Percentage of eosinophils in BALF. (C) OVA-specific IgE measured by ELISA in serum. (D and E) IL-4 and IL-10 in lung supernatants, measured by ELISA. (F and G) A total of 5×10^5 $CD4^+CD25^+$ or $CD4^+CD25^-$ cells either from OVA or PBS INT mice were transferred as described above. (F) Mononuclear cell subpopulations number in BALF of recipient mice after challenge. (G) Flow cytometry analysis of $CD4^+CD25^-mTGF\beta^+$ expression in BLN cells of donor mice after challenge. (H) Asthmatic donor mice were tolerized (OVA INT) or not (PBS INT). $CD4^+CD25^-mTGF\beta^{+/-}$ T cells were purified from lungs and BLN collected at day 52. Recipient asthmatic mice received 5 x 10E5 $CD4^+CD25^-mTGF\beta^{+/-}$ T cells from donor mice at day 35. BALF were collected after OVA challenge (aerosols) at day 43 and absolute number of eosinophils, macrophages and lymphocytes in BALF were determined. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

inflammatory than BALF from animals transferred with mTGFβ-depleted $CD4^+CD25^-$ T cells, as expressed by total inflammatory cell number and eosinophil number (Fig. 6H). This strongly indicated that $CD4^+CD25^-mTGF\beta^+$ T cells played a key role in the induction of intranasal tolerance to OVA. Nonetheless, the depletion of $CD4^+CD25^-mTGF\beta^+$ T cells only led to partial reversion of tolerance, suggesting that $mTGF\beta^+$-expressing $CD4^+CD25^-$ T cells may not be the sole partners involved in this phenomenon.

$CD4^+CD25^+$ T cells are necessary for the induction of $CD4^+CD25^-$ T cells with regulatory activity

As previously shown, the deletion of $CD4^+CD25^+$ T cells prior to INT with OVA completely abrogated the induction of tolerance (24). To verify the hypothesis that $CD4^+CD25^+$ T cells (>80% $Foxp3^+$) were indispensable intermediates in the mechanisms leading to the induction of $CD4^+CD25^-$ T cells with regulatory properties, OVA-sensitized mice were treated with anti-CD25 mAb PC61 or with PBS as control at D31 prior to INT with OVA, then at D38 and D45 and finally re-exposed to OVA aerosols (Fig. 7A). Mice were sacrificed at D52 and $CD4^+CD25^-$ T cells purified by magnetic cell sorting. $CD4^+CD25^-$ T cells from PC61 pre-treated or control animals were then transferred to OVA-sensitized/OVA aerosol challenged mice at D35. Transferred animals were re-exposed to OVA aerosols and BALF analysed for inflammatory cell count (Fig. 7B). There was no significant difference between non-transferred control animals and CD25-depleted/$CD4^+CD25^-$ T cell-transferred animals. In contrast, BALF from mice transferred with $CD4^+CD25^-$ T cells from CD25 non-depleted animals were significantly less inflammatory as measured by eosinophil count. This strongly suggested that $CD4^+CD25^+$ T regulatory cells were indispensable for induction of tolerance and required for $CD4^+CD25^-$ T cells education into cells with regulatory properties.

Induction of tolerance via the nasal route also occurs in the absence of IL-10 expression

To evaluate the role of IL-10 in the induction of tolerance, we took advantage of the C57BL/10 IL-$10^{-/-}$ knockout model (Fig. 8). After sensitization with OVA and exposure to

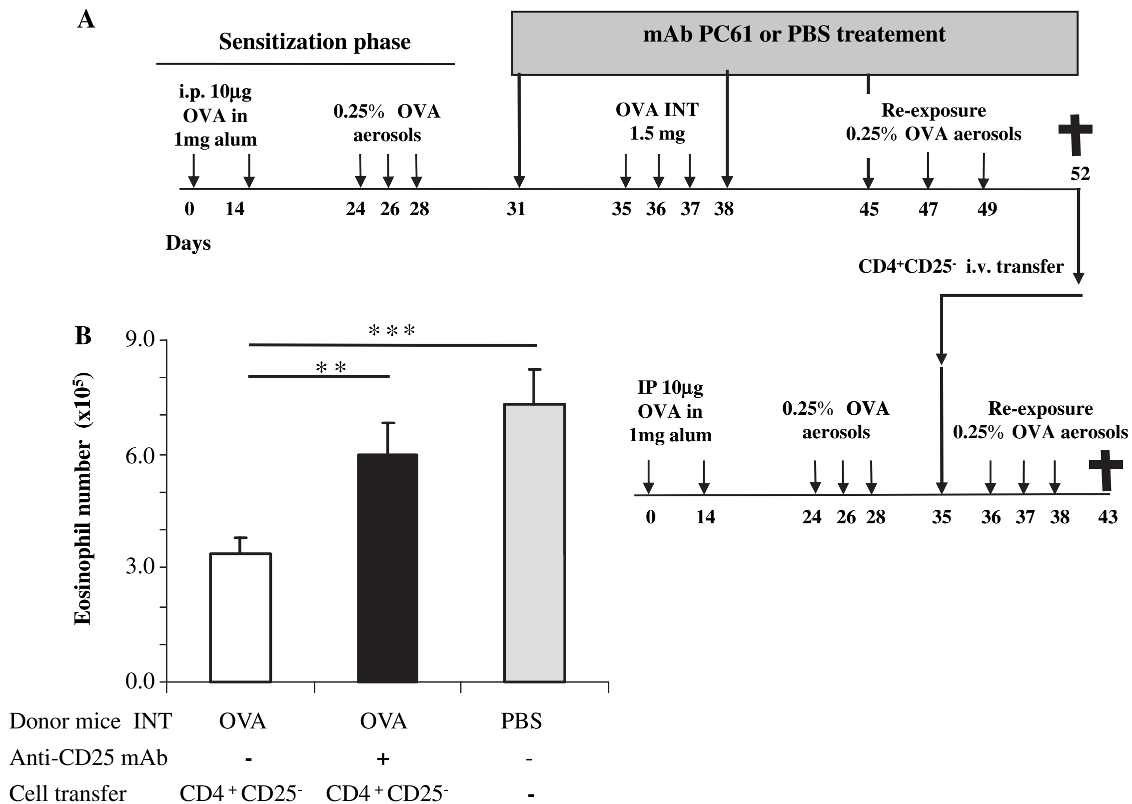

Fig. 7. CD4⁺CD25⁺ T cells are necessary for CD4⁺CD25⁻ T cells to acquire regulatory properties. (A) Donor BALB/c mice were sensitized to OVA, intranasally treated (INT) with 1.5 mg OVA or PBS every day for 3 days. Donor mice were treated or not with anti-CD25 mAb PC61 (three i.p. injections of 1 mg at days 31, 38 and 45). Donor mice were challenged with OVA 0.25% aerosols and sacrificed at day 52. CD4⁺CD25⁻ T cells were purified from lungs and BLN from donor mice at day 52 and transferred into recipient mice at day 35. OVA-sensitized recipient mice were transferred with CD4⁺CD25⁻ T cells at day 35, challenged with OVA aerosols at days 36, 37 and 38 and sacrificed at day 43. (B) Number of eosinophils in BALF as a function of the various conditions were described under (A). Crosses indicate sacrifice. $^{**}P < 0.01$; $^{***}P < 0.001$.

OVA aerosols, mice were treated intranasally either with OVA or with PBS as described in Fig. 1. As demonstrated in the BALB/c IL-10$^{+/+}$ model, a marked down-regulation of total inflammatory cells and eosinophils in BALF was observed not only in C56BL/10 IL-10$^{+/+}$ mice (Fig. 8A and C) but also in C56BL/10 IL-10$^{-/-}$ mice (Fig. 8B and D) upon INT with OVA and re-exposure to OVA aerosols at D57. There was no significant difference in BALF infiltration between IL-10$^{-/-}$ mice and IL-10$^{+/+}$ mice. This strongly suggested that IL-10 may not play a unique role in the down-regulation of inflammatory infiltrate in BALF in this model and especially in the down-regulation of eosinophil recruitment.

Discussion

We demonstrated in this study the ability of nasal application of OVA to protect against further re-exposure to OVA aerosols in a therapeutic model of asthma. Mucosal delivery of OVA via the nasal route markedly reduced eosinophil and other inflammatory cell recruitment into BALF and bronchial airways, as well as AHR, in an antigen-specific manner. T_h2 and T_h1 cytokines were strongly inhibited, and OVA-specific T cell proliferative response was markedly decreased, establishing a state of tolerance to OVA. Protection upon re-exposure to OVA was sustained up to 32 days after nasal application of allergen. Moreover, tolerance to allergen re-exposure could be transferred by CD4⁺CD25⁻ T cells from tolerized mice. *In vivo*, in the absence of CD4⁺CD25⁺(Foxp3⁺) T cells, CD4⁺CD25⁻ T cells from tolerized mice were in contrast unable to acquire regulatory properties and to transfer tolerance. A significant fraction of tolerogenic CD4⁺CD25⁻ T cells expressed mTGFβ, which appeared to play a significant role in the induction of tolerance in contrast to IL-10.

Taken together, our data supported a down-regulation of both T_h1 and T_h2 cytokines rather than a strict T_h2 to T_h1 shift, an effect compatible with observations done in models of allergen SIT (30, 31) and in venom immunotherapy (32). These results also demonstrated the potential of mucosal delivery of allergen to efficiently down-regulate an established bronchial inflammation and to improve asthma. In this regard, our observations are in line with a previously published model demonstrating the superior capacity of intranasal immunotherapy over intradermal immunotherapy in down-regulating airway allergic inflammation (13). However, in contrast to our approach, Takabayashi *et al.* were not able to down-regulate T_h2 inflammation at a systemic level. The higher doses of OVA used in our model may account for this difference. Furthermore, in our approach, INT with OVA was not only able to induce long-term protection (Fig. 2) but also

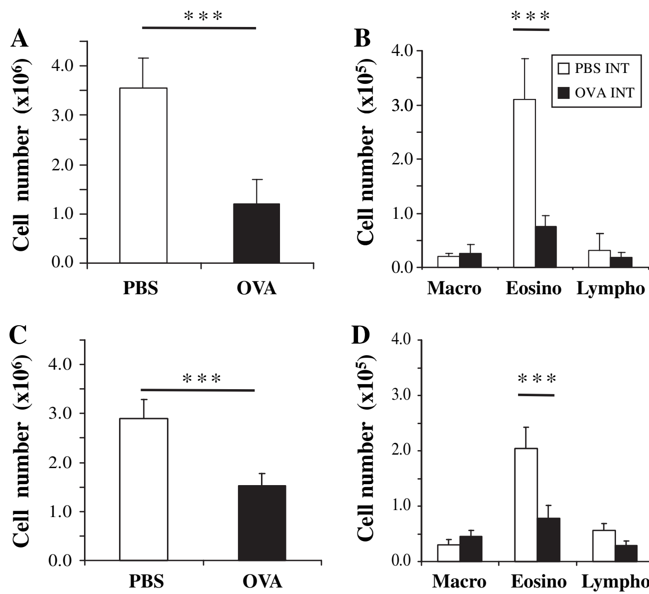

Fig. 8. Induction of tolerance in IL-$10^{-/-}$ mice. C57BL/10 (wild type) (A and B) and C57BL/10 IL-$10^{-/-}$ (C and D) mice were sensitized to OVA as described in Fig. 1, namely with two i.p. injections of 10 μg alum-adsorbed OVA and three 0.25% OVA aerosols. Ten days later, mice were intranasally treated with 1.5 mg OVA or PBS for controls and challenged three times with OVA 0.25% aerosols at days 50, 52 and 54. BALF were harvested at day 57. (A and C) Total cell in BALF expressed as cell number. (B and D) Mononuclear cell subpopulations in BALF expressed as cell number. ***$P < 0.001$.

resistance to a second series of allergen challenges. This sustained effect of INT with OVA strongly suggested that a long-term suppressive memory response was taking place, in agreement with the long-term protection provided by subcutaneous immunotherapy in mouse (33) and in human (8).

INT with OVA abrogated the rise in OVA-specific IgE present in control mice after re-exposure, whereas in contrast IgG1 increased. Similarly, after intranasal but not oral tolerance in a prophylactic murine model of asthma, T_h2 cytokine secretion by allergen-specific T cells were down-regulated although IgG1 increased concomitantly (34).These findings were globally in agreement with allergen SIT to respiratory or hymenoptera allergens in human, associated with an increase in serum allergen-specific IgG1, IgG4 and IgA (5, 35, 36). *In vitro*, mouse IgG1 were able to block human IgE interaction with allergen by competing on similar Bet v 1 epitopes (37). IgG1 may thus take part to the protection observed in OVA INT animals upon re-exposure to allergen.

At D47, i.e. 1 week after OVA INT, we observed a marked enhancement of the inflammatory response in lungs and BLN, in particular of the T_h2 cytokine response (Figs 4 and 5). Eosinophil recruitment into BALF was strongly stimulated with the lowest dose of OVA (0.5 mg) as compared with the highest dose (1.5 mg) (Fig. 1), although both treatment levels led to significant and comparable impairment in eosinophil recruitment upon OVA aerosol challenges at D57. These results are reminiscent of previous mice and human immunotherapy studies demonstrating that prior to tolerance induction, a transient inflammatory phase may occur (12, 36, 38). Lung inflammation and T-cell activation in the early phase of nasal allergen SIT appears therefore a pre-requisite to immune response down-regulation.

The mechanisms underlying the down-regulation of the allergic airway response in our model still need to be fully characterized, but at that stage, our data suggest a role for both $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells. Indeed, transfer of $CD4^+$ T cells from OVA INT mice reproduced the effect of INT with OVA (Fig. 6). Moreover, protective effects were fully reproduced by the transfer of $CD4^+CD25^-$ T cells and only when isolated from mice treated intranasally with OVA. This strongly suggested that nasal application of OVA was able to induce a subset of $CD4^+$ T cells able to transfer tolerance, i.e. to express regulatory properties. Our observations are reminiscent of previous reports in models of autoimmunity, where the *in vivo* transfer of $CD4^+CD25^-$ T cells was able to induce protection (39–41). However, this does not exclude a role for $CD4^+CD25^+$ T cells as recently demonstrated (21, 23). Importantly, although we were not able to directly transfer tolerance with $CD4^+CD25^+$ T cells (>80% $Foxp3^+$) (24), we showed here that in the absence of $CD4^+CD25^+Foxp3^+$ T cells, $CD4^+CD25^-$ T cells (>95% $Foxp3^-$) were unable to acquire regulatory properties (Fig. 7). How $CD4^+CD25^+Foxp3^+$ natural T regulatory cells exert their 'educative' action on induced $CD4^+CD25^-$ $Foxp3^-$ T regulatory cells is still unclear. Our data support a potential role for TGFβ, a key regulatory cytokine involved in the protection upon re-exposure to allergen (42, 43). This was strongly suggested by the markedly enhanced production of TGFβ in lung supernatants *ex vivo* (Fig. 4D), although we cannot rule out that part of this TGFβ secretion may also be due to non-immune cells (44). Furthermore, we know from our previous publication that $CD4^+CD25^+Foxp3^+$ T cells express high level of mTGFβ, suggesting a role for mTGFβ in the induction of $CD4^+CD25^-$ with regulatory properties (24). This interpretation has been previously supported by a model of aerosolized antigen-induced tolerance involving cell–cell contact with regulatory $CD4^+$ T cells that co-express mTGFβ and Foxp3 (20). A synergy between mTGFβ and Cytotoxic T-Lymphocyte Antigen 4 in T regulatory cells-mediated suppression of proliferation of $CD25^-$ effector T cells has been previously demonstrated (45). Furthermore, INT with OVA is associated with a significant enhancement of the expression of mTGFβ on $CD4^+CD25^-$ T cells (Fig. 6). Again, mTGFβ appears to play here also a key role since the exclusion by magnetic cell sorting of $CD4^+CD25^-mTGF\beta^+$ T cells from $CD4^+CD25^-$ T cells transferred to asthmatic recipients significantly enhanced eosinophil recruitment into BALF. Exclusion of mTGFβ cells, however, did not fully restore lung inflammation suggesting the contribution of other anti-inflammatory mechanisms. In this respect, the question is still open whether the induction of $CD4^+CD25^-$ tolerogenic T cells we observed (induced T regulatory cells) may be further converted into $CD4^+CD25^+Foxp3^+$ T cells (42).

The anti-inflammatory role of IL-10 is in contrast debatable in our model. Indeed, IL-10 production in lung homogenates or in supernatants from BLN and lung cell culture rose markedly after INT with OVA but sharply dropped after

re-exposure to OVA aerosols (Fig. 5). These findings correlated with an increased frequency of $CD4^+$ IL-10^+ T cells in lungs and BLN after INT with OVA contrasting with a marked decrease after OVA challenges. Our data may possibly be representative of IL-10 production by T regulatory cells as suggested in prophylactic models (16, 19) but may also result from IL-10 production by OVA-specific T_h2 effector cells (46). Furthermore, tolerance induction to OVA was obtained in IL-$10^{-/-}$ mice, although in a different genetic background, showing that IL-10 was not unique in the process leading to tolerance. These data were in agreement with our previous observations indicating that the frequency of $CD4^+CD25^-$IL-10^+ T cells from tolerized mice was low (as compared with $CD4^+CD25^+$ T cells) and did not significantly differ from the frequency of $CD4^+CD25^-$IL-10^+ T cells in non-tolerized animals (24). We cannot, however, fully exclude a possibly redundant anti-inflammatory role for IL-10 or an activity on other parameters than those considered here (lung eosinophilia, IgE production). We indeed showed an enhanced production of IL-17 in the lungs of tolerized animals (24), a result that may suggest a regulatory contribution of this cytokine in turning down the T_h2 immune response of asthmatic mice in the absence of concomitant enhancement of the T_h1 response (47). On the one hand, IL-17 induction in tolerized mice may support an immunoregulatory activity of IL-17 in dampening the T_h2 airways inflammation (47–49). On the other hand, IL-17 secretion into the lungs would be a logical consequence of the concomitant action of TGF_β and IL-6 leading to the generation of T_h17 cells (50). A better understanding of the IL-17 response and involvement in tolerance induction is ongoing in our laboratory.

Taken together, our model of tolerance in asthma contributed to demonstrate that INT with OVA led to the induction of $CD4^+CD25^-$ T cells with regulatory properties under the control of $CD4^+CD25^+Foxp3^+$ T cells. The fine mechanisms leading to the generation of T regulatory cells appeared to involve mainly TGFβ/mTGFβ expression as key players, the respective roles of which will have to be further analysed. This model also strongly supports allergen-specific induction of tolerance based on a mucosal approach in human with allergic asthma.

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Disclosure

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