

Intranasal treatment with ovalbumin but not the major T cell epitope ovalbumin 323–339 generates interleukin-10 secreting T cells and results in the induction of allergen systemic tolerance

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Summary

Background Nasal administration of major peptide T cell epitopes gives contradictory data on the induction of peripheral tolerance.

Objective To compare the prophylactic effect of intranasal treatment (INT) on the development of an allergic response, using either ovalbumin (OVA) or its major T cell epitope OVA 323–339 (OVAp).

Methods BALB/c mice were treated intranasally with OVA or OVAp and subsequently immunized s.c. with OVA. Anti-OVA-specific antibody, T cell proliferation and cytokine responses were analysed. In an adoptive transfer model using OVAp specific TCR transgenic (Tg) T cells from D011.10 mice, *in vivo* tracking and characterization of transferred T cells in the cervical, inguinal and bronchial lymph nodes (BLN) and in the spleen were determined by FACS analysis.

Results Prophylactic INT with OVA induced T cell tolerance towards subsequent OVA s.c. immunizations, inhibiting OVA specific T cell proliferation, IgE and IgG1 production, in contrast to INT with OVAp, which was unable to induce tolerance. *In vivo* analysis of transferred OVA-specific TCR Tg T cells showed that INT with OVA induced a preferential activation of T cells in BLN, as opposed to a broad, systemic activation with OVAp. *In vivo*, OVAp INT led to faster and more sustained cell division cycles than OVA INT. *Ex vivo*, tolerance to OVA was associated with the generation of IL-10 secreting CD4⁺ T cells in BLN of OVA-treated mice only.

Conclusion INT with OVA but not with OVAp led to regional (as opposed to systemic) T cell activation and the induction of IL-10 secreting CD4⁺ T cells in BLN, potentially critical steps in the induction of T cell-specific tolerance via the nasal route.

Keywords allergy, anergy, IL-10, immunodominant, immunotherapy, intranasal, ovalbumin, peptide, regulatory, tolerance

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Introduction

Short immunodominant peptides derived from allergens have been advised to decrease the risk of anaphylaxis inherent in conventional-specific immunotherapy. This approach was successful in various degrees with restricted sets of immunodominant peptides derived from major allergens of cat danders (Fel d 1) or bee venom (phospholipase A2) in humans [1, 2], or with peptides from Fel d 1 and birch pollen major allergen Bet v 1 in mice [3–5]. Intranasal administration of the immunodominant house dust mite (HDM) allergen Der p 1 in primed mice reduced T cell response not only to the epitope, but also to the entire protein via the so-called linked

immunosuppression [6]. However, though these studies were promising, conflictual data were also published. Subcutaneous immunization with the major T cell epitope-containing peptide of the HDM Der p 2 elicited a strong immune response rather than a state of tolerance [7]. In a murine model of asthma, immunotherapy with ovalbumin (OVA) reduced airway eosinophilia and hyper-responsiveness, whereas immunotherapy with the immunodominant OVA peptide 323–339 (OVAp) enhanced them [8].

Induction of tolerance to OVA via the nasal or conjunctival routes using OVA has been demonstrated in several studies [9–11]. However, it is not clear whether tolerance can be obtained via the nasal mucosa using the immunodominant peptide OVAp in a prophylactic setting. In this study, we analysed the prophylactic effect of intranasal treatment (INT), with either whole OVA or the immunodominant T cell epitope OVA 323–339 (OVAp) on the development of a

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systemic allergic response to OVA. Early cellular events and potential generation of regulatory T cells were further characterized in both settings.

Materials and methods

Animals

Four-week-old female BALB/c mice (H-2^d) were obtained from Harlan Olac (Zeist, the Netherlands) and used at the age of 6–8 weeks. DO11.10 TCR transgenic (Tg) mice were originally produced by Murphy et al. [12], and were a kind gift of Dr J.-C. Sirard (ISREC, Epalinges, Switzerland).

Peptides and proteins

OVA was obtained from Flucka (Buchs, Switzerland). OVAp (KKKISQAVHAAHAEINEAGRKKK) was synthesized and purified as described elsewhere [13]. Lysines were added to increase OVAp (ISQAVHAAHAEINEAGR) solubility at high concentration. Both peptides showed similar dose-response curves when tested in a proliferation assay with OVAp specific T cells (data not shown).

INT and systemic immunization protocol

Mice were lightly anaesthetized with halothane (Halocarbon B.P., Arovet A.G., Zollikon, Switzerland). As indicated in each experiment, OVA or OVAp (100, 400, 800, 1000 or 2000 µg) in 20 µL phosphate-buffered saline (PBS) was administered intranasally for 3 consecutive days (days 0, 1 and 2). Control mice received PBS only. Mice were then immunized every 10 days (days 16, 26, 36 and 46) by s.c. injection of 2 µg of alum (1 mg)-adsorbed OVA. One day before each injection, serum samples were collected to establish the kinetics of IgE, IgG1 and IgG2a antibody response. Mice were killed at day 52 for T cell proliferation and cytokine assay.

Isolation, carboxyfluorescein diacetate succinimidyl ester (CFSE) labelling and adoptive transfer of KJ1-26⁺CD4⁺T cells

Spleen and lymph nodes of DO11.10 mice were collected and processed into single cell suspension. Cell suspensions were depleted of CD19⁺ and CD8⁺ cells, using anti-CD19⁺ and anti-CD8⁺ microbeads according to manufacturer's protocol (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Prior to adoptive transfer, KJ1-26⁺CD4⁺ T cell subpopulation in the resulting CD4⁺ T cells was determined by FACS analysis. CFSE (Molecular probes, Eugen, OR, USA) labelling was performed as described [14]. At day 0, 2–4 × 10⁶ KJ1-26⁺CD4⁺ T cells were injected into the vein of the tail following previous protocols [15]. Animals were subsequently treated intranasally with OVA 1 mg or OVAp 1 mg (or PBS as control) as described above (days 1, 2 and 3). An early cellular analysis was performed at day 7, a later one at day 17 or 21, 4 days after a single s.c. immunization with alum-adsorbed OVA (2 µg).

Antibody isotype determination in sera

Anti-OVA IgE, IgG1 and IgG2a responses were titrated by ELISA. Plates were coated overnight at 4 °C with 50 µL of OVA (5 µg/mL in carbonate/bicarbonate buffer pH 9.6) or with a purified rat anti-mouse IgE (2 µg/mL; clone R35-72, BD PharMingen, San Diego, CA, USA) (for standardization), and blocked in 1% bovine serum albumin (BSA)/PBS for 2 h at 37 °C. Ten by ten dilutions of sera in 1% BSA/0.05% Tween were incubated for 2 h at 37 °C. Purified IgE, used as standard (clone R27-74, BD PharMingen) was diluted as indicated by the manufacturer. After washing, biotinylated rat anti-mouse IgE mAb (clone R35-92, BD PharMingen) or biotinylated rabbit anti-mouse IgG1 or IgG2a mAbs (Caltag, San Francisco, CA, USA) were added for 30 min at 37 °C. Assays were revealed by the addition of alkaline phosphatase-conjugated ExtrAvidin (1:10 000) for 30 min at 37 °C, and finally of *p*-nitrophenylphosphate (Sigma Chemical Co., Buchs, Switzerland). Optical density was determined at 405 nm on a microtitre plate analyzer (MR5000, Dynatech Laboratories, Embrach-Embraport, Switzerland).

Flow cytometry

Single cell suspensions were prepared and incubated with anti-FcγRII/III mAb (24G2), anti-CD4-PE mAb (RM4-5), biotinylated anti-CD62L mAb (Mel-14), biotinylated anti-CD25 mAb (PC61), or anti-CD11c-APC mAb (HL3), rat anti-mouse-PerCP, biotinylated mouse anti-rat, biotinylated rat anti-mouse, streptavidin APC, streptavidin-PerCP (all from BD PharMingen), FITC-conjugated or purified clone-type-specific KJ1-26 mAb (Caltag Laboratories, Burlingame, CA, USA). Annexin V analysis was performed using the Annexin V-FITC Apoptosis detection kit, according to manufacturer's protocol (BD PharMingen). Samples were analysed on a FACScalibur and fluorescence intensity was determined using CellQuest software (Becton Dickinson, Mountain View, CA, USA). For cell sorting, labelled cells were sorted on an FACS Vantage SE (Becton Dickinson). Purity was >95%.

Proliferation assay

2 × 10⁵ lymph node cells or 5 × 10⁵ splenocytes were seeded in a 96-well flat-bottomed plate with the indicated antigen concentrations in a total volume of 200 µL DMEMc (DMEM, Gibco-BRL, Life technologies, AG, Basel, Switzerland) supplemented with 50 µM β-mercaptoethanol (Fluka, Buchs, Switzerland), 2 mM L-glutamine, 1 mM sodium-pyruvate, 1 mM HEPES, 100 IU/mL penicillin-streptomycin (Gibco-BRL) and 10% FCS (Gibco-BRL). After 64 h, 1 µCi ³H-Thymidine (Hartmann Analytic GmbH, Braunschweig, Germany) was added for the last 18 h. Cells were then harvested and the incorporated ³H-Thymidine counted in a β-counter (Top Count, Packard Bioscience S.A., Zürich, Switzerland).

Cytokine secretion

One day after the last INT, the spleen and bronchial lymph nodes (BLN) were collected and processed into single cell suspension. 5 × 10⁶ splenocytes or BLN lymphocytes

were stimulated *in vitro* in the presence or absence of OVA 250 µg/mL or OVAp. Ninety-six hours later, supernatants were collected and cytokine content determined by sandwich ELISA. IFN- γ was measured using the capture mAb R4-6A2, the biotinylated detection mAb XMGI.2 (all from BD PharMingen). Standard purified recombinant IFN- γ was used as indicated by the manufacturer (BD PharMingen). IL-10 was measured using the capture mAb JES052A5 and the recommended biotinylated detection mAb (all from R&D GmbH, Wiesbaden-Nordenstadt, Germany). Standard recombinant mouse IL-10 was used as indicated by the manufacturer (BD PharMingen). IFN- γ and IL-10 ELISA were performed as described by the manufacturers (BD PharMingen and R&D, respectively).

Cytokine mRNA expression

Cytokine mRNA expression was determined by RT-PCR. Splenocytes (4×10^6) were stimulated for 27 h *in vitro* with 250 or 10 µg of OVA or OVAp, respectively, in a total volume of 1 mL DMEMc. Cells were then harvested and total RNA extracted as described elsewhere [16]. Quality of RNA was determined by agarose gel and quantified by optical density at 260/280 nm. For FACS sorted cells, RNA was extracted using RNeasy[®] kit (Qiagen, Basel, Switzerland) according to manufacturer's protocol. One to two micrograms of total RNA were reverse transcribed in a total volume of 30 µL containing 0.5 µg oligodT (Amersham Pharmacia Biotech, Dübendorf, Switzerland), $1 \times$ first strand buffer (Gibco-BRL), 10 mM DTT (Gibco-BRL), 500 µM dNTPs (Pharmacia), 80 U Superscript II reverse transcriptase (Gibco-BRL). RT mixture was incubated for 50 min at 42 °C, followed by 4 min at 95 °C. The resultant cDNA was stored at -20 °C until PCR amplification. The final PCR mixture contained 2–4 µL cDNA, $1 \times$ PCR buffer (Qiagen), 200 µM dNTPs (Pharmacia), 1 U Taq polymerase Hot start (Qiagen), 0.2 µM of each sense and anti-sense primers and sterile water in a

volume of 25 µL. The thermal cycling parameters were 94 °C for 15 min, followed by 35 cycles each of denaturation (94 °C for 50 s), annealing (60 °C for 50 s) and extension (72 °C for 1 min). A final extension of 72 °C for 4 min was included. Cytokine-specific primers were synthesized by Microsynth (Balgach, Switzerland) according to sequences described elsewhere [17]. PCR products were visualized by UV light after migration on 1.2% agarose gel containing ethidium bromide. Quantification was performed using 'Advanced American Biotechnology Software, Quanytify1D, advanced and compared'. Calculations were performed as follows: the intensity of cytokine PCR product for each cDNA was corrected for the related GAPDH PCR product intensity. Then the relative fold increase was calculated as a ratio of corrected cytokine PCR product of stimulated cells/corrected cytokine PCR product of non-stimulated cells.

Statistical analysis

Differences between groups were analysed by one-way ANOVA using Student–Newman–Keuls multiple comparisons test on an Instat 3.05 software. $P < 0.05$ was considered as the limit of confidence (95% confidence interval).

Results

Prophylactic INT with OVA prevents IgE and IgG1 responses, and induces T cell hyporesponsiveness to OVA

INT with OVA significantly prevented, in a dose-dependent manner (100, 400, 1000, 2000 µg), the specific IgE (Figs 1a and b) and IgG1 (Figs 1c and d) rise induced in PBS-treated control group by s.c. immunization with OVA. IgG2a production was unchanged in contrast (data not shown). In parallel, INT with moderate (100, 400 µg, data not shown) or

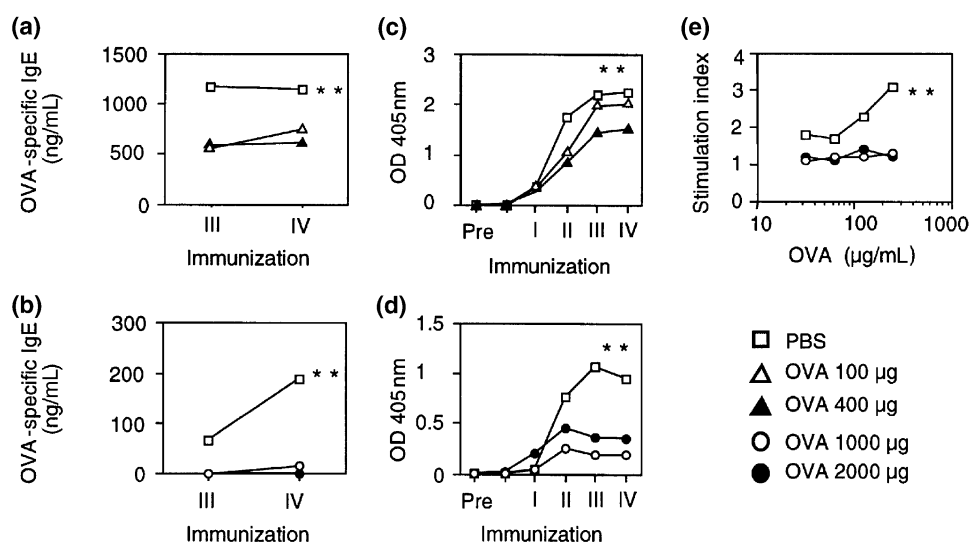


Fig. 1. Prophylactic intranasal treatment with OVA prevents IgE and IgG1 responses, and induces T cell hyporesponsiveness to OVA. Mice were treated intranasally at days 0, 1 and 2 with OVA or PBS as control, and immunized s.c. with alum-adsorbed OVA (2 µg) at day 16 (I), 26 (II), 36 (III) and 46 (IV). (a, b) OVA-specific IgE, (c, d) OVA-specific IgG1, (e) OVA-specific T cell proliferation at day 52. Results are expressed as the mean of five individual mice per conditions. Significant differences between control and active groups are indicated by * $P < 0.05$ and ** $P < 0.01$ (Student–Newman–Keuls multiple comparisons test). OVA, ovalbumin; PBS, phosphate-buffered saline.

high doses of OVA (1000, 2000 μ g, Fig. 1e) abrogated the proliferative capacity of OVA-specific splenocytes. IL-4 and IFN- γ mRNA expression in splenocytes was evaluated by RT-PCR after *in vitro* restimulation with OVA. As expected, control mice exhibited an IL-4/IFN- γ ratio (1.4) in favour of a Th2-type of cytokine response. In contrast, INT with OVA 1000 μ g resulted in a shift towards a Th1 pattern with an IL-4/IFN- γ ratio of 0.5.

Prophylactic INT with OVAp was unable to induce tolerance to OVA

Whatever the doses of OVAp used, there was no difference in IgE (Fig. 2a) and IgG1 (data not shown) production between peptide-treated groups and control mice. However, IgG2a production reached its peak more quickly in the peptide groups (Fig. 2b, 400, 800 μ g). T cell proliferation in response to OVA was unaffected by OVAp INT at day 52 after four s.c. immunizations with alum-adsorbed OVA (Fig. 2c). Degradation and/or non-absorption of the peptide was ruled out, since mice prophylactically treated with OVAp 1000 μ g and sensitized with alum (1 mg)-adsorbed OVAp 10 μ g (days 16, 26, 36 and 46) exhibited a reduced specific T cell proliferative response to OVAp at day 52 (Fig. 2d), demonstrating that OVAp could indeed induce anergy towards its own epitope. Consistently with the absence of down-regulation of the IgE and IgG1 responses, OVAp INT enhanced the IL-4/IFN- γ ratio up to 2.6 (1.4 in control group), a modulation compatible with a reinforcement of the Th2 response.

Early and late tracking of KJ1-26⁺CD4⁺ T cells in response to OVA or OVAp INT in lymphoid organs

To approach the mechanisms leading to the opposed effects of OVA vs. OVAp on tolerance induction, we first tracked KJ1-26⁺CD4⁺ T cells in lymphoid organs. KJ1-26⁺CD4⁺ T cells were adoptively transferred in mice, subsequently treated intranasally with OVA, OVAp or PBS as control (Fig. 3). Very consistently, with a peak at day 4, OVA INT induced a sustained and preferential increase (4–19-fold increase, range of three independent experiments) in KJ1-26⁺CD4⁺

T cells in BLN, whereas there was only a 2–5- and 1–3-fold increase in the spleen and inguinal lymph nodes (ILN), respectively (Fig. 3a as a representative experiment). In contrast, OVAp INT induced a marked, systemic increase in KJ1-26⁺CD4⁺ T cells (5–10-fold) in all organs tested. At later time-points (day 21), 4 days after a first s.c. immunization with alum-adsorbed OVA 2 μ g at day 17, the proportion of KJ1-26⁺CD4⁺ T cells burst as expected in ILN (4–12-fold as compared with day 7) of control animals to stabilize, after further OVA immunizations at days 33 and 45, at percentages constantly superior to OVA-treated mice (Fig. 3b) or to non-immunized mice (10–20-fold higher, data not shown). In contrast, in OVA-treated group, despite repeated immunizations, there was no increase in KJ1-26⁺CD4⁺ T cells neither in ILN nor in BLN. The marked differences in KJ1-26⁺CD4⁺ T cell percentages observed after OVA vs. OVAp INT in ILN, cervical lymph nodes (CLN) and in the spleen were not due to enhanced apoptosis in the OVA group, since Annexin V⁺ KJ1-26⁺CD4⁺ T cells were present in equal proportion in both treatment conditions at day 4 or 7. In addition, propidium iodide staining showed similar percentages of dead/necrotic TCR Tg T cells in OVA- or OVAp-treated groups (data not shown).

Kinetics of KJ1-26⁺CD4⁺ T cell proliferation in response to OVA or OVAp INT in lymphoid organs

Kinetics of cell division differed markedly between groups. At an early time-point (Fig. 4a, day 7), whereas after OVAp INT more than 95% (95–100%, range of three independent experiments) of transferred CFSE-labelled KJ1-26⁺CD4⁺ T cells had undergone at least one division cycle in BLN, ILN or in the spleen, 54–70% were still undivided in control group. This contrasted with the OVA-treated group, which actively divided in BLN (91–95%), but significantly less in ILN or in the spleen. Indeed, upon OVA INT, no more than 46% of CFSE-labelled KJ1-26⁺CD4⁺ T cells entered into >6 cycles of cell divisions in tested lymphoid organs, as compared with more than 75% upon OVAp INT. At a later time-point (Fig. 4b, day 17), 4 days after s.c. immunization with alum-adsorbed OVA 2 μ g, CFSE-labelled KJ1-26⁺CD4⁺ T

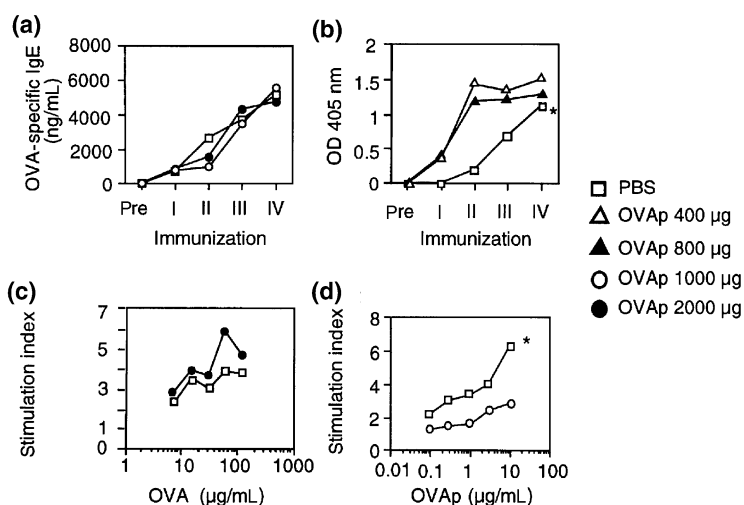


Fig. 2. Prophylactic intranasal treatment with OVAp is unable to induce tolerance to OVA. Mice were treated with OVAp vs. PBS as described in Fig. 1. (a) OVA-specific IgE, (b) OVA-specific IgG2a, (c) specific T cell proliferation in response to OVA, (d) specific T cell proliferation at day 52 in response to OVAp in mice treated with OVAp 1000 μ g and sensitized with alum (1 mg)-adsorbed OVAp 10 μ g at days 16, 26, 36 and 46. Results are expressed as mean of five individual mice per conditions. Significant differences between control and active groups are indicated by * $P < 0.05$ (Student–Neuman–Keuls multiple comparisons test). OVAp, OVA peptide 323–339; OVA, ovalbumin; PBS, phosphate-buffered saline.

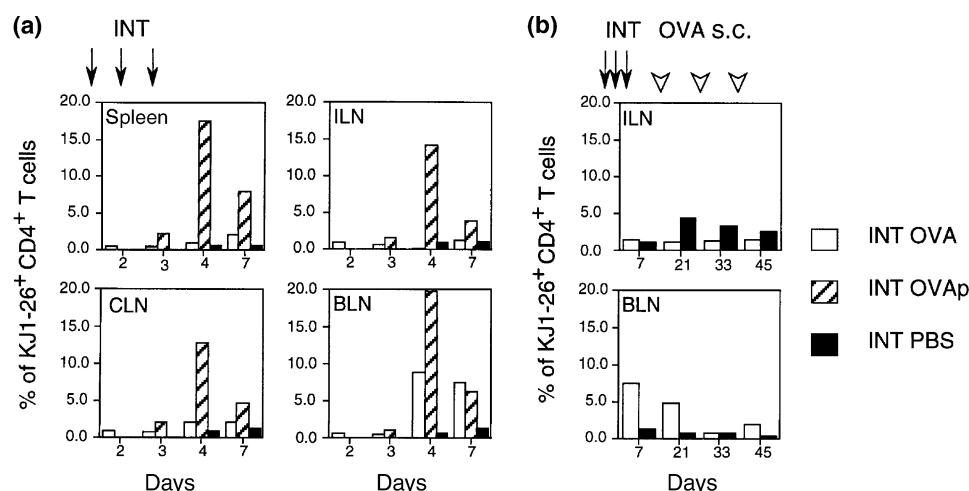


Fig. 3. Early and late tracking of KJ1-26⁺CD4⁺ T cells in response to OVA or OVAp INT in lymphoid organs. KJ1-26⁺CD4⁺ T cells ($2-5 \times 10^6$) were transferred at day 0. Mice were treated intranasally with OVA, OVAp or PBS at days 1, 2 and 3. KJ1-26⁺CD4⁺ T cell percentage in total CD4⁺ T cells was analysed (a) at days 2, 3, 4 and 7 after transfer, (b) 4 days after s.c. immunization with alum-adsorbed OVA (2 µg) at days 21, 33 and 45, in the spleen, ILN, CLN and BLN. Data (means) are representative of two independent experiments performed on two (PBS group), respectively three (OVA, OVAp groups) individual mice per group. Day 7 time-point was tested with six individual mice for all groups; at day 7, between-group statistical analysis (OVA vs. OVAp): $P < 0.05$, for spleen, ILN and CLN; $P > 0.05$ in BLN. OVA, ovalbumin; OVAp, OVA peptide 323–339; INT, intranasal treatment; PBS, phosphate-buffered saline; ILN, inguinal lymph nodes; CLN, cervical lymph nodes; BLN, bronchial lymph nodes.

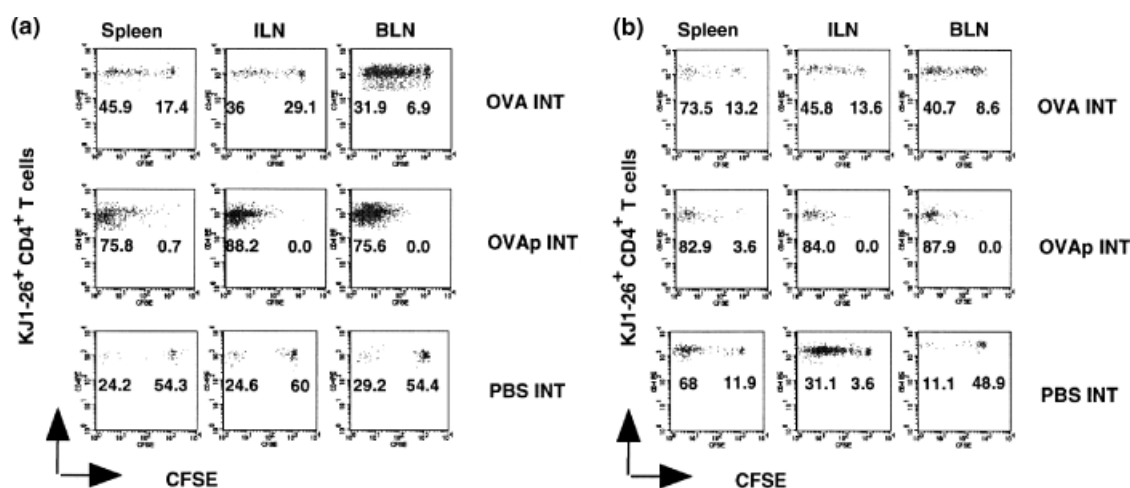


Fig. 4. Kinetics of KJ1-26⁺CD4⁺ T cell proliferation in response to OVA or OVAp INT in lymphoid organs. *In vivo* analysis of KJ1-26⁺CD4⁺ T cell divisions in the spleen, ILN, CLN and BLN, (a) at day 7 after transfer with $2-5 \times 10^6$ CFSE-labelled KJ1-26⁺CD4⁺ T cells, (b) at day 17, 4 days after a first immunization with alum-adsorbed OVA (2 µg). Numbers indicate the percentage of undivided cells (CFSE high) or cells with > 6 cell division cycles (CFSE low). Data are representative of two independent experiments performed on two (PBS group), respectively three (OVA, OVAp groups) individual mice per group. OVA, ovalbumin; OVAp, OVA peptide 323–339; INT, intranasal treatment; ILN, inguinal lymph nodes; CLN, cervical lymph nodes; BLN, bronchial lymph nodes; CFSE, carboxyfluorescein diacetate succinimidyl ester; PBS, phosphate-buffered saline.

cells divided actively in control mice (undivided cells $< 30\%$ in the spleen, $< 20\%$ in ILN). In contrast, the number of undivided cells did not markedly change in OVA-treated group. Undivided CFSE-labelled KJ1-26⁺CD4⁺ T cells from OVAp group were practically undetectable.

Prophylactic OVA INT induces IL-10-producing T cells

Twenty-four hours after the last INT with OVA, OVAp or PBS, CD4⁺CD11c[−] T cells were isolated from BLN by cell sorting (purity $> 96\%$), and RT-PCR performed *ex vivo* for

IL-10 and IFN- γ transcripts. IL-10 mRNA expression was 2–3-fold higher after OVA INT than after OVAp or PBS INT (Fig. 5a). Two- to threefold higher IFN- γ mRNA transcripts were also induced after OVA INT as compared with OVAp-treated mice (Fig. 5b). These data were confirmed by measuring IL-10 secretion into supernatants of total BLN lymphocytes cultured for 96 h in the presence of OVA (Fig. 5c). IL-10 secretion was markedly stimulated in BLN in OVA-treated group, but not in OVAp-treated group (ratio IL-10 OVA/IL-10 OVAp: 4.7). Although at that time-point, both conditions induced comparable secretion of IFN- γ in

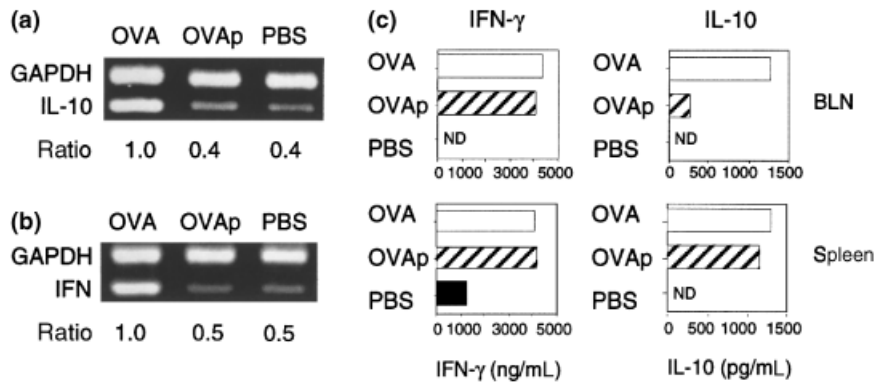


Fig. 5. Prophylactic OVA INT induces IL-10-producing T cells. Naïve mice were subjected to INT with OVA, OVAp or PBS. (a) IL-10 and (b) IFN- γ mRNA expression in CD4⁺-CD11c⁺ T cells (sorted from BLN) was performed by RT-PCR after *ex vivo* RNA extraction 24 h after the last INT. PCR products were quantified using an AAB software as described in Materials and methods. (c) Total lymphocytes from BLN and the spleen, isolated 24 h after the last INT, were stimulated for 96 h with OVA 250 μ g/mL. IL-10 and IFN- γ were measured by ELISA in cell supernatant. Data are representative of three independent experiments on pools of three mice per group. OVA, ovalbumin; INT, intranasal treatment; OVAp, OVA peptide 323–339; PBS, phosphate-buffered saline; BLN, bronchial lymph nodes; ND, not detectable.

BLN, the ratio of IFN- γ /IL-10 were markedly opposed (3.5 in OVA group vs. 15.3 in OVAp group). IL-10 and IFN- γ secretion did not differ in the spleen in contrast.

Discussion

In this study, we examined the ability of prophylactic INT with OVA as opposed to its major epitope OVAp to prevent the development of a systemic response to the allergen. Prophylactic INT with OVA-induced T cell tolerance to OVA as demonstrated by (1) OVA-specific T cell hyporesponsiveness and (2) Th1 immune deviation with abrogation of OVA-specific IgE and IgG1 rise in response to sensitization to OVA. In contrast, the major OVA epitope, OVAp, was unable to induce tolerance towards subsequent OVA s.c. immunizations, whatever the doses applied on the nasal mucosa. These results are in contrast with the full T cell tolerance obtained by intranasal administration of an immunodominant peptide from Der p 1 via linked immunosuppression [6, 18]. Several hypotheses could explain these discrepant results. Indeed, antigens as well as experimental protocols differ markedly. The HDM allergen Der p 1 is a small protein of about 20 kDa. It contains a quasi unique T cell epitope, which largely dominates the T cell response. OVA in contrast is a 42 kDa protein, which contains multiple T and B cell epitopes. Some of them, from cryptic, may become immunogenic during the process of tolerance induction. Indeed, a cryptic peptide of Der p 2 revealed itself a potent immunogen when used for immunotherapy [7]. Moreover, OVAp contains a nested set of at least three overlapping T cell epitopes [19]. Tolerance to each of them might not be achievable using this particular peptide, because TCR contact residues will vary depending on the epitope recognized and may make it difficult to tolerize the bulk T cell response in this manner. Intrinsic biochemical and/or physicochemical properties of the tolerogenic peptide do not most probably play a crucial role in its capacity to induce tolerance, as suggested by comparison of peptides used in several studies [6, 8, 20, 21]. All, including ours, are not only potential tolerogen, but are also capable of stimulating specific T cells. Second, when the pI of peptides such as those derived from Bet v 1, Der p 1 or OVA are compared,

those pI appear to be highly different (4.5 for Bet v 1; 6.0 for OVAp; 8.2 for Der p 1_{111–132}) and unrelated to their final tolerogenic or activating capability toward their respective protein. Finally, the intrinsic activity of some allergens, such as the cysteine protease activity of Der p 1, is most likely not playing any role, since peptides lose this property. However, one cannot exclude unique competences of some peptides, such as the Der p 1 peptide, which appears to be able to induce a bystander tolerance towards unrelated proteins [22]. There is so far no final explanations to the discrepancies observed, and indeed in several studies in the field of autoimmunity or asthma, a large range of peptide-dependent immunomodulatory effects were observed, from lack of tolerance to specific linked suppression through non-specific bystander suppression [8, 23, 20].

At least three mechanisms may underlie the induction of mucosal tolerance: clonal deletion, clonal anergy or the generation of a suppressive/regulatory T cell population. Dose of antigen, duration of treatment and route of administration are crucial factors that determine which of these mechanisms may be involved [24–29]. In the present study, we intended to determine the conditions and the mechanisms underlying on the one hand induction of tolerance by OVA, on the other hand systemic activation by the immunodominant OVA peptide, although both were administered via the nasal route. There was no difference in terms of mucosal airway response, since within the first 48 h bronchoalveolar lavage fluid cell count and cytokine content (IL-4, IL-5, IFN- γ) were undistinguishable from those of control group (data not shown). OVA-specific TCR Tg T cell response was, however, drastically different in the two approaches, with a very active T cell proliferation and a largely systemic T cell distribution to all lymphoid organs tested upon OVAp INT, contrasting with a predominantly localized response in BLN upon OVA INT (Fig. 3). Differences in T cell distribution to lymphoid organs were not related to programmed cell death, which was active at the same level in OVA, OVAp and control groups (data not shown). It could not be explained by differences in kinetics of cell distribution. Indeed, peak levels of KJ1-26⁺CD4⁺ T cells occurred simultaneously in OVA- or OVAp-treated groups in early as well as late time-points (Figs 3a and b). As previously shown [10], we confirmed that upon OVA INT T cell hyporesponsiveness was preceded by active cell division, but

this occurred preferentially in BLN (Fig. 4). In OVAp-treated mice at that time-point, T cell divided even more actively and rapidly, but in a systemic pattern. As compared with control animals, OVA-specific T cell hyporesponsiveness was clearly demonstrated by the *quasi* absence of response to OVA s.c. immunization in the OVA-treated group late in the course of INT (day 17) (Fig. 4b). The minimal number of cell divisions noted at day 17 can most probably be ascribed either to a residual post-INT activation or to homeostatic regulation. In a therapeutic model of bronchial inflammation in contrast, improvement of asthma after s.c. OVA was related to the induction of a strong, synchronized and systemic T cell response, whereas INT of mice with OVA or OVAp resulted in asthma degradation, and was associated to a weak non-synchronized T cell response [24]. We reproducibly showed here that induction of allergic tolerance via the nasal route in naïve mice could indeed be induced with OVA, and involved a mainly regional T cell response in BLN. These discrepancies might be related to large technical differences between protocols including approaches in naïve mice vs. sensitized animals, doses and site of OVA administration as well as timing of the observations. Globally, these two sets of observations underline that tolerance may proceed via markedly different mechanisms in naïve vs. sensitized animals. Our data are consistent with those obtained in other models of tolerance induction via the nasal route using OVA or Bet v 1 [10, 21], as well as a therapeutic approach in PLA2-sensitized mice [30].

Ex vivo, IL-10-positive T cell expansion was taking place only in BLN of OVA, but not OVAp-treated mice, nor in controls. These results are in agreement with recent data demonstrating the *in vivo* generation of IL-10 secreting CD4⁺ T cells in the spleen of animals treated intranasally with OVA [31]. *In vitro* generated IL-10 secreting T cells with dendritic cells from OVA-treated naïve animals were subsequently shown to have regulatory properties. T cell tolerance could be reversed by pretreatment with an anti-IL-10 mAb [31]. We add to this demonstration that IL-10 secreting T cells were generated in BLN, the preferential site of early specific T cell recruitment and division. This raised the possibility that OVA INT, via a mechanism not yet identified *in vivo*, would preferentially trigger the generation of T regulatory cells in BLN. In turn, these IL-10-secreting T cells, when stimulated in the periphery, may down-regulate a subsequent immune response by decreasing dendritic cells/APC function [32–34], or by directly inhibiting specific T cell proliferation [35, 36], resulting in a state of tolerance and T cell anergy. OVAp INT indeed was unable to induce the generation of IL-10-producing T cells in BLN, but was supporting in contrast a strong systemic specific T cell proliferation in all lymphoid organs tested. We postulate that the inability of OVAp to generate IL-10-producing T cells with potential regulatory properties in sufficiently high frequency may play a crucial role in the systemic T cell distribution to lymphoid organs induced by OVAp [37], as well as in the persistent proliferative response to OVA [38]. Alternatively, variation in T cell stimulation can be influenced by the nature of APC [39–41], antigen processing and presentation [42], antigen initial form [43] and avidity of T cell receptor engagement [44]. In this respect, professional APC such as dendritic cells play a dominant role in the initiation and regulation of the

immune response. Pathogen-specific T regulatory 1 cells were induced in the respiratory tract by a bacterial antigen (filamentous haemagglutinin) that stimulates IL-10 production by dendritic cells [45]. Furthermore, OVA INT was able to generate dendritic cells in BLN, which transiently secreted IL-10 [46]. We were not able to distinguish OVA vs. OVAp treated groups by their capacity to induce IL-10 secreting dendritic cells. IL-10 production by highly purified (>96%) CD11c⁺ dendritic cells was similar 24 h after nasal treatment in both conditions. Other factors such as dendritic cell subtype and time-course of IL-10 secretion by dendritic cells will have to be examined [47]. Similarly, though TGF- β has been involved in regulatory T cell functions in mice and humans [25–28], we were unable to demonstrate its production by spleen or BLN cells. Moreover, adhesion/homing molecule, chemokine and chemokine receptor expression are key players in the induction of tolerance [41, 48, 49]. Their roles, in particular, in BLN as the preferential site of tolerance induction in the intranasal OVA model, are currently under investigation.

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