Clinical and Experimental Allergy, 1–11 doi: 10.1111/j.1365-2222.2008.03056.x
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ORIGINAL PAPER

Toll-like receptor 2 agonist Pam3CSK4 enhances the induction of antigen-specific tolerance via the sublingual route

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Clinical and Experimental Allergy

Summary

Background Sublingual immunotherapy (SLIT) has been established in humans as a safe and efficacious treatment for type I respiratory allergies.

Objective In this study, we compared three Toll-like receptor (TLR) 2 ligands (Pam3CSK4, *Porphyromonas gingivalis* lipopolysaccharide and lipoteichoic acid) as potential adjuvants for sublingual allergy vaccines.

Methods These molecules were tested in co-cultures of adjuvant-pre-treated dendritic cells (DCs) with murine naïve $CD4^+$ T lymphocytes. Patterns of cytokine production, phenotype, proliferation and gene expression were analysed by ELISA, cytofluorometry and quantitative PCR, respectively. TLR2 ligands were subsequently tested in a model of SLIT in BALB/c mice sensitized with ovalbumin (OVA).

Results Among the three TLR2 ligands tested, the synthetic lipopeptide Pam3CSK4 is the most potent inducer of IL-12p35 and IL-10 gene expression in murine bone marrow-derived DCs, as well as in purified oral myeloid DCs. Only Pam3CSK4-treated DCs induce IFN-γ and IL-10 secretion by naïve $CD4^+$ T cells. Sublingual administration of Pam3CSK4 together with the antigen in BALB/c mice sensitized to OVA decreases airway hyperresponsiveness as well as OVA-specific T-helper type 2 (Th2) responses in cervical lymph nodes dramatically.

Conclusion Pam3CSK4 induces Th1/regulatory T cell responses, and as such, is a valid candidate adjuvant for sublingual allergy vaccines.

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Keywords adjuvant, allergy vaccines, Pam3CSK4, sublingual immunotherapy, tolerance induction

Submitted 14 January 2008; revised 21 April 2008; accepted 19 May 2008

Introduction

Allergen-specific immunotherapy is the only efficient causal treatment of type I respiratory allergies [1–3]. Allergy immunotherapy is known to reorient allergen-specific T cell responses from T-helper type 2 (Th2) to Th1 in atopic patients [4–8]. More recently, it has been shown that subcutaneous immunotherapy (SCIT) [9–11] and likely sublingual immunotherapy (SLIT) [12–15] also induce regulatory T cells (Tregs) capable of down-regulating Th2 responses through the production of IL-10.

In the present study, we focus on the sublingual route of immunization, which has been established as safe and efficacious in humans in order to induce allergen-specific tolerance [16–21]. Adjuvants are still needed to improve the efficacy of sublingual vaccines, most particularly as it applies to vaccines based on recombinant allergens. In this regard, there is a major interest in finding new adjuvants capable of inducing IFN-γ and/or IL-10 secretion by T cells. To this end, Toll-like receptors (TLRs) represent valid targets for candidates adjuvants, given their known involvement in the regulation of T cell responses [22, 23].

In this study, we compare the biological properties of three TLR2 ligands [Pam3CSK4, *Porphyromonas gingivalis* lipopolysaccharide (PgLPS) and lipoteichoic acid (LTA)]. Pam3CSK4 is a synthetic tripalmitoylated lipopeptide that mimics bacterial lipopeptides [24, 25]. PgLPS is a part of the cell wall of the Gram-negative bacterium *P. gingivalis* associated with periodontitis [26, 27]. LTA is a surface-associated molecule from Gram-positive bacteria that stimulates immune cells to produce pro-inflammatory cytokines [28].

Herein, we evaluate those molecules for their capacity to induce IFN-γ and/or IL-10 secretion by naïve $CD4^+$

T cells, when incubated with antigen-presenting cells *in vitro*. We confirm that among the TLR2 ligands tested, only Pam3CSK4 exhibits such unique immunomodulatory properties. In relation to this observation, Pam3CSK4 enhances antigen-specific tolerance induced by the sublingual route in a murine model of asthma.

Material and methods

Adjuvant testing on murine immune cells

BALB/c mice were bred in Stallergenes' animal facilities accredited by French authorities and handled by certified investigators. Bone marrow-derived dendritic cells (BMDCs) were generated as described elsewhere [29]. To test the effect of TLR2 ligands on cytokine production, 10^6 BMDCs were plated in a 24-well plate in 1 mL culture medium (RPMI 1640, supplemented with 10% fetal calf serum, antibiotics and 5×10^{-5} M β-2-mercaptoethanol, all from Invitrogen, Cergy Pontoise, France) in the presence of various concentrations (0.1, 1, 10 μg/mL) of either Pam3CSK4 (*N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-[*R*]-cysteinyl-[*S*]-seryl-[*S*]-lysyl-[*S*]-lysyl-[*S*]-lysyl-[*S*]-lysine × 3 HCl, EMC Microllections, Tuebingen, Deutschland), PgLPS (Invivogen, Toulouse, France) or LTA (Invivogen) for 24 h at 37 °C in 5% CO_2. Supernatants were harvested for cytokine production measurement. $CD4^+$ T cells from DO11.10 transgenic mice expressing a T cell receptor specific for peptide 323–339 from ovalbumin (OVA) were used to set up an OVA-specific BMDC/T co-culture. BMDCs were pulsed for 4 h with 25 μg/mL OVA, washed and treated for 24 h with 1 μg/mL of each of the TLR2 ligands. Cells were washed and 5×10^4 adjuvant-treated BMDCs were co-cultured in 500 μL complete culture medium with 5×10^5 $CD4^+$ T cells isolated from the spleen of DO11.10 mice using magnetic microbeads (Dynal, Oslo, Norway). Supernatants were recovered after either 1, 3 or 6 days of co-culture for cytokine production measurement. RNA was extracted from non-adherent cells to analyse the expression of Tbet, GATA3, Fox p3, IFN-γ, IL-4, TGF-β and IL-10 genes by quantitative real-time PCR. For carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution assays, DO11.10 $CD4^+$ T cells were labelled with CFSE (2 μM, Invitrogen) following the manufacturer's instruction.

To test the impact of candidate adjuvants on oral dendritic cells (DCs), sublingual/oral tissues (buccal floor and lingual tissues) were removed from naïve BALB/c mice and treated for 45 min at 37 °C with 400 U/mL collagenase type IV, 50 μg/mL DNase I (Roche Diagnostic, Mannheim, Germany) and 2 U/mL dispase (Invitrogen) in RPMI 1640 medium. Tissues were dissociated in phosphate-buffered saline (PBS) after adding 5 mM EDTA in PBS to block residual enzymatic activity. Cells obtained in suspension were subsequently stained, at 4 °C for 15 min, with anti-CD11b-PE, anti-CD11c-APC and anti-MHC class II-FITC monoclonal antibodies. A pure population of $CD11b^+$ $CD11c^-$ MHC class II^+ oral myeloid DCs was isolated with a MoFlo (Dako, Trappes, France) cell sorter. Cells were confirmed to be > 99% pure by flow cytometry re-analysis. Up to 10^4 $CD11b^+$ $CD11c^-$ MHC class II^+ cells per well were subsequently cultured for 24 h in 96-well U-bottom plates in 200 μL complete medium in the presence of either medium alone or 1 μg/mL Pam3CSK4. RNA was extracted as described below. For proliferation assays and cytokine production analysis, 1.5×10^3 $CD11b^+$ $CD11c^-$ MHC class II^+ oral DCs were treated for 24 h with Pam3CSK4 (1 μg/mL) or medium. Oral DCs were subsequently cultured with 3×10^4 CFSE-labelled $CD4^+$T DO11.10 cells for 3 days in the presence of OVA peptide 323–339 ($OVA_{323-339}$, 1 μg/mL, Peptide International, Louisville, KY, USA).

Measurement of cytokine production and gene expression

The production of IL-5, IL-10 and IFN-γ was evaluated by ELISA (R&D Systems, Lille, France). IFN-γ and IL-10 production in supernatants harvested from oral DC/T co-cultures was measured using a cytometric bead array Flex Set (BD Biosciences, Rungis, France).

Analysis of intracellular cytokine production was performed on BMDC/T co-cultures. T cells were stimulated with 1 μg/mL plate-bound anti-CD3 (555273, BD Biosciences) for 4 h, and GolgiPlug (BD Biosciences) was added during the last 2 h. Cells were surface-stained with anti-CD4 (11B11) and anti-CD25 antibodies (PC61, all from BD Biosciences), and then fixed and permeabilized using the Intraprep reagent (Beckman Coulter, Fullerton, CA, USA). Cells were subsequently washed and incubated with PE-labelled anti-IL-10 (JES5-16E3) and FITC-conjugated anti-IFN-γ antibodies (XMG1.2, all from BD Biosciences). Cell fluorescence was measured using an FC500 Flow Cytometer (Beckman Coulter).

Analysis of mRNA expression was carried out by real-time PCR using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) as described in [29].

Therapeutic sublingual immunotherapy in BALB/c mice sensitized to ovalbumin

The capacity of candidate adjuvants to enhance SLIT efficacy was tested in a therapeutic *in vivo* model of OVA-induced asthma [30]. Briefly, BALB/c mice were sensitized with OVA by two intraperitoneal injections (10 μg OVA + 2 mg alum) at days 0 and 14 and then challenged with OVA by aerosol (1% w/v in PBS) delivered on days 21–24. For desensitization purposes, endotoxin-free OVA (always < 0.1 EU/μg protein) was prepared [31]. Two sublingual treatments (500 μg OVA per dose with or without 10 μg candidate adjuvant diluted in a final volume of 50 μL PBS) were performed each week for 8 weeks (Fig. 1).

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Groups of eight sensitized mice received either PBS as a negative control, OVA alone, Pam3CSK4, PgLPS, OVA plus Pam3CSK4 or OVA plus PgLPS. After sublingual immunization, mice were challenged during 2 consecutive days with a 1% w/v OVA aerosol. After 24 h, airway hyperresponsiveness (AHR) was evaluated by measuring enhanced pause (Penh) by whole-body plethysmography (Buxco) as described elsewhere [30]. The Penh index, expressed as an increase relative to the baseline airway resistance, was obtained as a ratio between Penh values measured after exposure to inhaled metacholine (50 mg/mL) and after inhalation of nebulized PBS, as described previously (Fig. 1) [30].

Adoptive T cell transfer and in vivo *proliferation assays*

To assess the impact of sublingual administration of adjuvants on T cell responses *in vivo*, 5×10^6 CFSE-labelled DO11.10 $CD4^+$ T cells were adoptively transferred by a retroorbital intravenous injection into BALB/c mice. One day after, four groups of four BALB/c mice received either PBS, OVA (500 μg), Pam3CSK4 (10 μg) or Pam3CSK4 plus OVA sublingually in 50 μL PBS. Seven days later, cervical lymph nodes were removed and pooled within each group of mice. OVA-specific transferred T cells were stained with the anti-clonotypic PE-KJ1.26 antibody (BD Biosciences) directed against the transgenic TCR expressed by T cells in DO11.10 mice. Proliferating T cells were evaluated by FACS analysis using an FC500 Flow Cytometer (Beckman Coulter) as cells with a decreased CFSE-associated fluorescence.

Statistical analysis

Penh values of mice treated sublingually with OVA with or without an adjuvant or levels of gene expression and cytokines produced by immune cells were compared using Student's *t*-test. A *P*-value <0.05 was considered to be statistically significant.

Results

Effect of Toll-like receptor 2 agonists on dendritic cell maturation and cytokine production

After 24 h incubation, Pam3CSK4 and PgLPS (1 μg/mL each) up-regulate the expression of both MHC class II (IA/IE), CD40 and CD80, molecules at the surface of murine BMDCs to a level comparable with LPS (1 μg/mL), whereas LTA has a more limited effect (data not shown). When measuring cytokine gene expression by quantitative PCR, we observed that all TLR2 ligands up-regulate the expression of both IL-12p35 and IL-10 genes by murine DCs. Pam3CSK4 consistently elicits a significant up-regulation of IL-10, IL-12p35 and TNF-α gene expression in comparison with PgLPS and LTA (Fig. 2a). The production of TNF-α and IL-12p40 cytokines by BMDCs is markedly increased after treatment with either Pam3CSK4 or PgLPS, but not with LTA (Fig. 2b). Only Pam3CSK4 triggers a clear secretion of the regulatory cytokine IL-10 by BMDCs.

Pam3CSK4-treated bone marrow-derived dendritic cells stimulate interferon-γ and interleukin-10 production by murine naïve $CD4^+$ T cells

We further tested the capacity of adjuvant-treated BMDCs to prime OVA-specific T cells. To this end, OVA-specific $CD4^+$ T cells obtained from DO11.10 mice were co-cultured with BMDCs pulsed with OVA (10 μg/mL) in the presence of TLR2 ligands (1 μg/mL). DCs were washed twice before using them in the co-cultures to preclude any direct effect of TLR2 ligands on T lymphocytes. BMDCs treated with the adjuvant in the absence of OVA were used

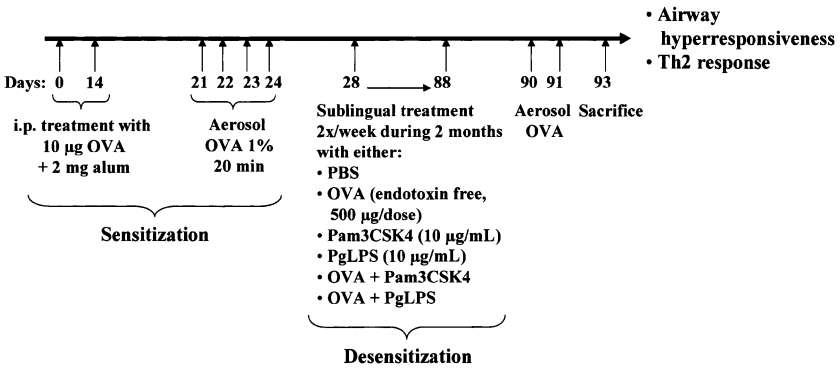

Fig. 1. Murine model of OVA-induced asthma and sublingual immunotherapy. BALB/c mice were sensitized to OVA as described in 'Material and methods'. For sublingual desensitization, mice (eight per group) were divided into groups receiving either OVA (500 μg/dose), Pam3CSK4 or PgLPS alone (10 μg/dose), OVA plus adjuvant or PBS alone as a control. Mice were treated twice a week for 2 months applying 50 μL solutions under the tongue with a micropipette, while holding animals on their back for 1 min to prevent swallowing. OVA, ovalbumin; PgLPS, *Porphyromonas gingivalis* LPS; PBS, phosphate-buffered saline.

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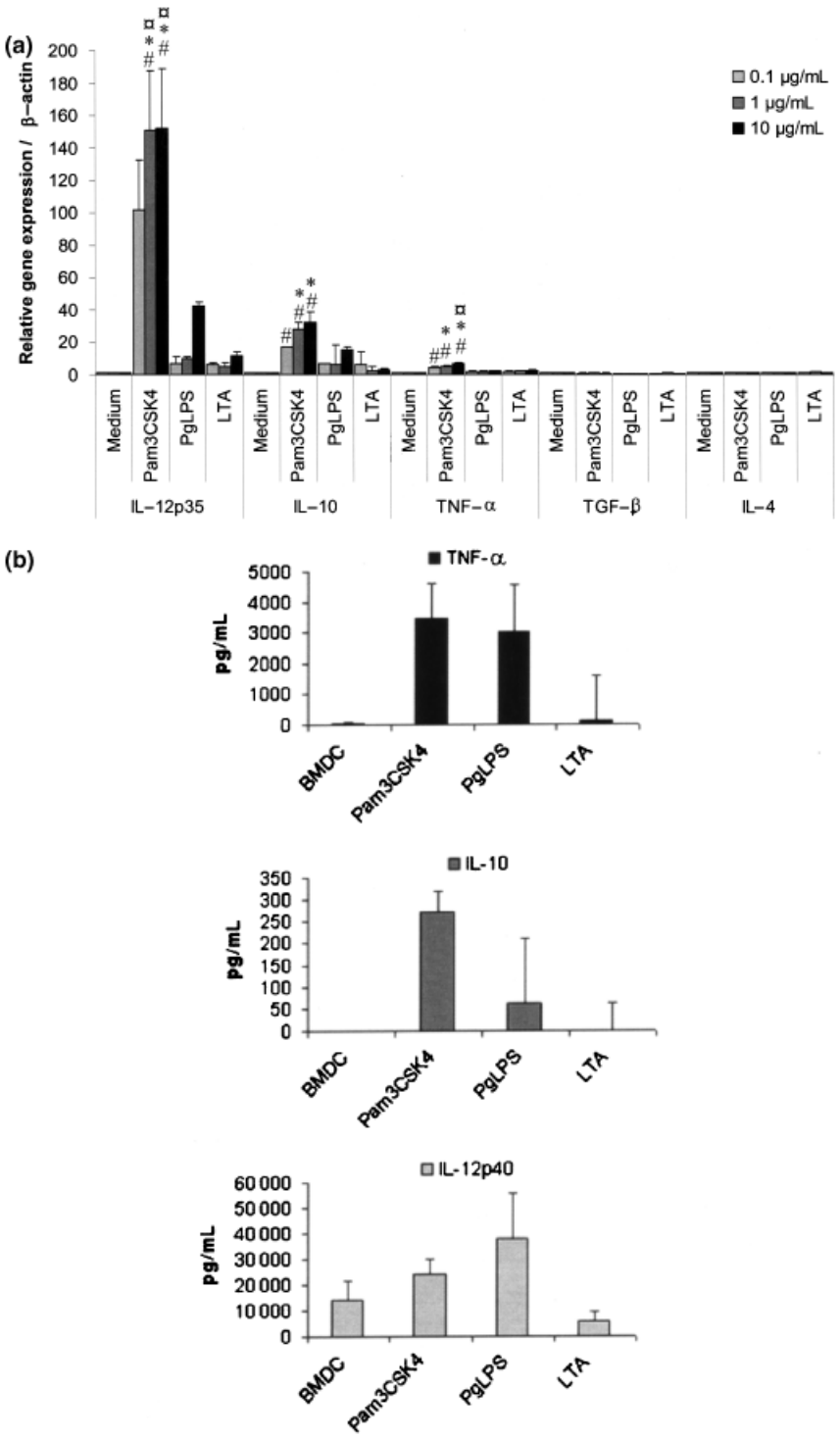

Fig. 2. BMDCs cultured with Pam3CSK4 produce TNF-α, IL-10 and IL-12p40 cytokines. (a) BMDCs were incubated for 24 h in the presence of 0.1, 1 or 10 µg/mL of either Pam3CSK4, PgLPS or LTA. Gene expression was evaluated by real-time PCR. Data shown are mean of five independent experiments ± SEM. $^{\#}P$-value < 0.05 for Pam3CSK4 vs. medium; $^{*}P$-value < 0.05 for Pam3CSK4 vs. LTA; $^{¤}P$-value < 0.05 for Pam3CSK4 vs. PgLPS. (b) Cytokine production of BMDCs treated with 1 µg/mL TLR2 ligands was measured by ELISA. Data shown are the mean ± SEM of three independent experiments. IL-12p70 was not detected in culture supernatants in any of these experiments. BMDCs, bone marrow-derived dendritic cells; PgLPS, *Porphyromonas gingivalis* lipopolysaccharide; LTA, lipoteichoic acid; TLR, Toll-like receptor.

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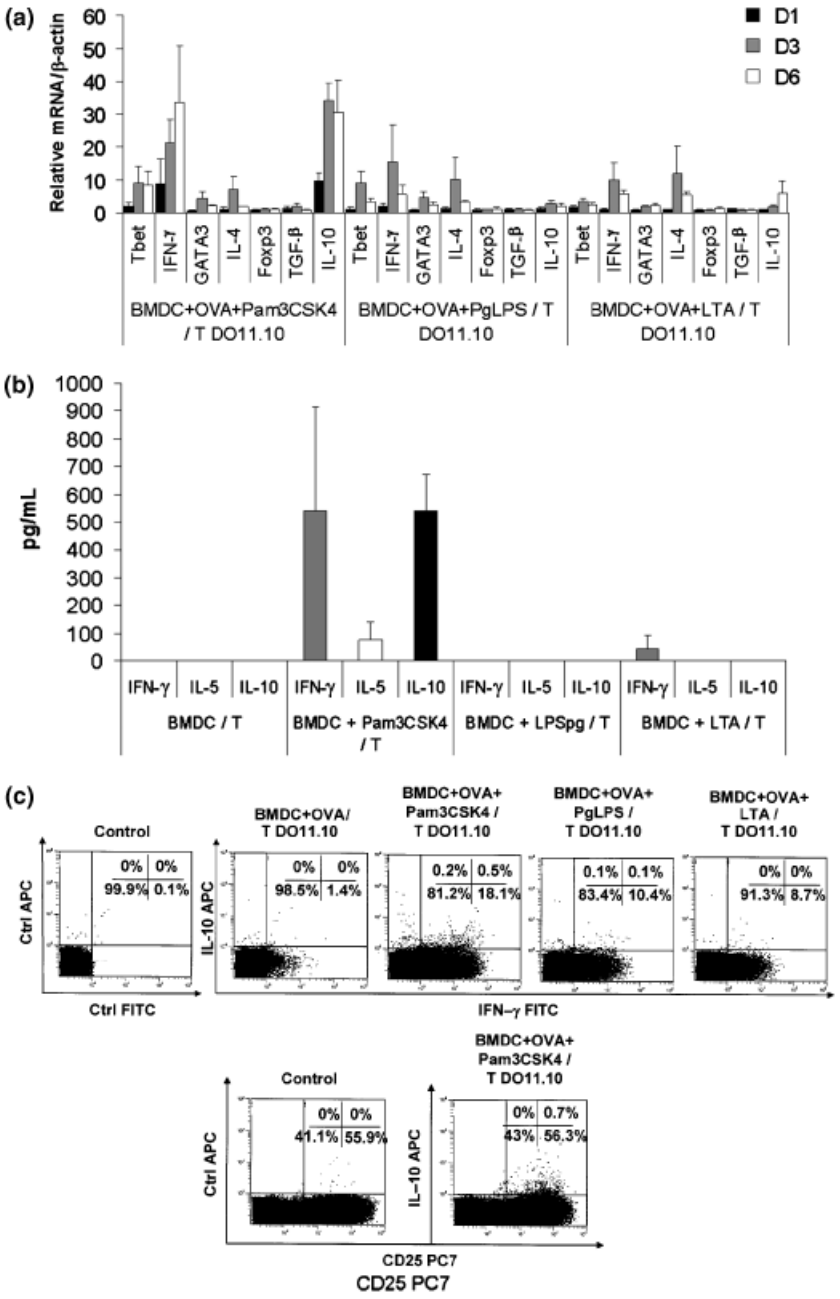

Fig. 3. Pam3CSK4-treated BMDCs induce IFN-γ and IL-10-secreting $CD4^+$ $CD25^+$ T cells. (a) After pre-incubation with OVA (25 μg/mL) and then TLR2 ligands (1 μg/mL), BMDCs were washed and co-cultured with OVA-specific DO11.10 $CD4^+$ T cells using a DC : T ratio of 1 : 10. Gene expression was evaluated by real-time PCR after 1, 3 or 6 days of co-culture. Data normalized to the amount of β-actin are shown as the mean ± SEM of three independent experiments compared with the negative control (i.e. untreated BMDCs cultured with $CD4^+$ T cells) arbitrarily set to 1. (b) Cytokine production was measured in culture supernatants by ELISA after 3 days of co-culture. Data shown are the mean ± SEM of three independent experiments. (c) Cytokine production by T cells cultured for 3 days with TLR2 ligand-treated BMDCs (1 μg/mL) was analysed by intracytoplasmic staining. Cells are gated on $CD4^+$ T cells. Results are representative of two independent experiments. BMDCs, bone marrow-derived dendritic cells; OVA, ovalbumin; TLR, Toll-like receptor.

as a negative control. We monitored the expression of various genes encoding transcription factors or cytokines associated with either Th1, Th2 or Treg patterns of differentiation. As shown in Fig. 3a, co-culture with Pam3CSK4-treated BMDCs induces a strong (i.e. over a 30-fold) increase in IFN-γ and IL-10 gene expression by naïve $CD4^+$ T cells after 3 and 6 days. In contrast, co-culture with PgLPS or LTA-treated DCs up-regulates IFN-γ but not IL-10 gene expression in naïve $CD4^+$ T lymphocytes. ELISA measurement of cytokines secreted in

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culture supernatants confirms that only Pam3CSK4-treated DCs elicit the production of IFN-γ and IL-10 by murine $CD4^+$ T cells (Fig. 3b). Intracytoplasmic co-staining further demonstrates that IL-10-secreting $CD4^+$ cells are uniformly $CD25^+$ and secrete IL-10 alone or together with IFN-γ (Fig. 3c). In these experiments, neither gene expression nor cytokine production was detected under control conditions without OVA as well as in cell cultures lacking T lymphocytes (data not shown). This confirms that the response observed is due to OVA-specific $CD4^+$ T cells.

Antigen presentation to OVA-specific T cells in the presence of TLR2 ligands was further analysed in a CFSE dilution assay (Fig. 4). BMDCs pulsed with OVA induce a strong proliferation of OVA-specific $CD4^+$ T cells, with 77.3% proliferating T cells. BMDCs incubated with either PgLPS or LTA in the presence of OVA did not affect T cell proliferation. Interestingly, when cultured in the presence of BMDCs treated with either 10 or 1 μg/mL of Pam3CSK4, the percentage of proliferating T cells decreased in a dose-dependent manner to 22.6% and 47.4%. No proliferation was observed under control conditions performed without OVA. Although not shown, no differences were observed with any of the TLR2 ligands in terms of induction of Fox p3 expression in either proliferating or non-proliferating T cells. The capacity of $CD4^+$ T cells from the primary co-culture to inhibit the proliferation of naïve $CD4^+$ T cells in response to OVA was tested in a functional assay. No increase in the suppressive activity was observed in T cells obtained from co-cultures with BMDCs treated with either one of the TLR2 ligands (data not shown).

The unique IFN-γ- and IL-10-inducing capacity of Pam3CSK4 was confirmed on human immune cells. When cultured with Pam3CSK4-treated monocyte-derived DCs (MoDCs), the relative mRNA level of IFN-γ and IL-10 genes in human allogeneic $CD4^+$ naïve T cells is significantly increased (data not shown). Neither IFN-γ nor IL-10 gene induction was detected in human T cells stimulated with MoDCs treated with either PgLPS or LTA.

Therapeutic sublingual administration of ovalbumin plus Pam3CSK4 reduces airway hyperresponsiveness

Given the specific immunomodulatory profile of Pam3CSK4, we tested its capacity to induce tolerance in an established model of allergic asthma in BALB/c mice (Fig. 1). In this model, OVA-sensitized animals develop a readily detectable AHR in response to metacholine and a severe lung inflammation associated with cellular infiltrates and mucus hyperproduction [30]. These animals also exhibit a strong Th2 immune response (with antigen-specific IL-5 and IL-10 production in lymphoid organs) and high levels of OVA-specific IgG1 and IgE seric antibodies [30]. Sensitized animals (eight per group) were treated sublingually twice a week for 2 months with OVA (500 μg/dose) with or without Pam3CSK4 (10 μg/dose) or PgLPS (10 μg/dose) administered in 50 μL PBS (Figs 5a and b). When

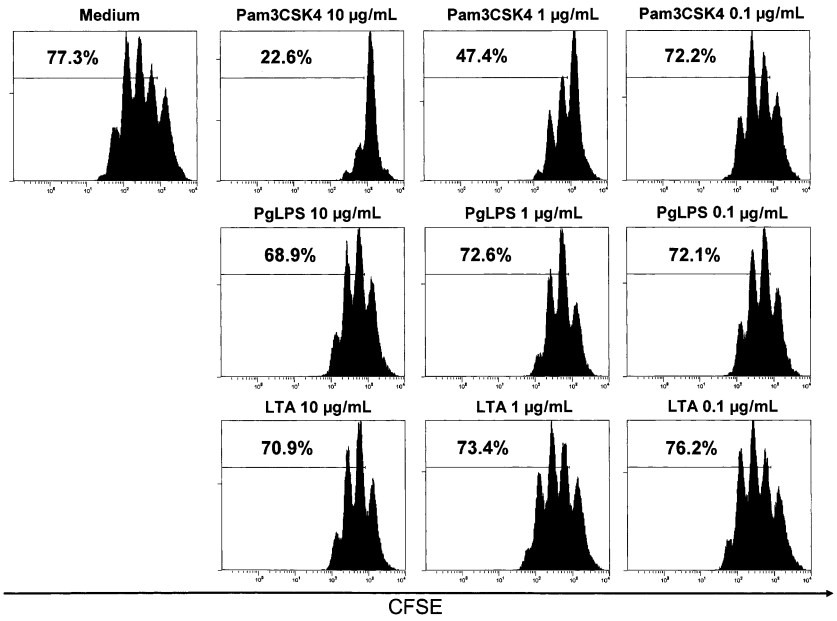

Fig. 4. T cell proliferation following stimulation with BMDCs treated with TLR2 ligands. The proliferation of $CD4^+$ OVA-specific T cells cultured for 3 days with OVA (10 μg/mL) plus BMDCs treated with either Pam3CSK4, PgLPS or LTA (at 10, 1 or 0.1 μg/mL) was measured in a CFSE dilution assay. Less than 5% proliferating T cells were observed in the presence of BMDCs without OVA (not shown). Cell viability, assessed by trypan blue dye exclusion, was always >90%. Results are representative of three independent experiments. BMDCs, bone marrow-derived dendritic cells; OVA, ovalbumin; TLR, Toll-like receptor; CFSE, carboxyfluorescein diacetate succinimydil ester; PgLPS, *Porphyromonas gingivalis* lipopolysaccharide.

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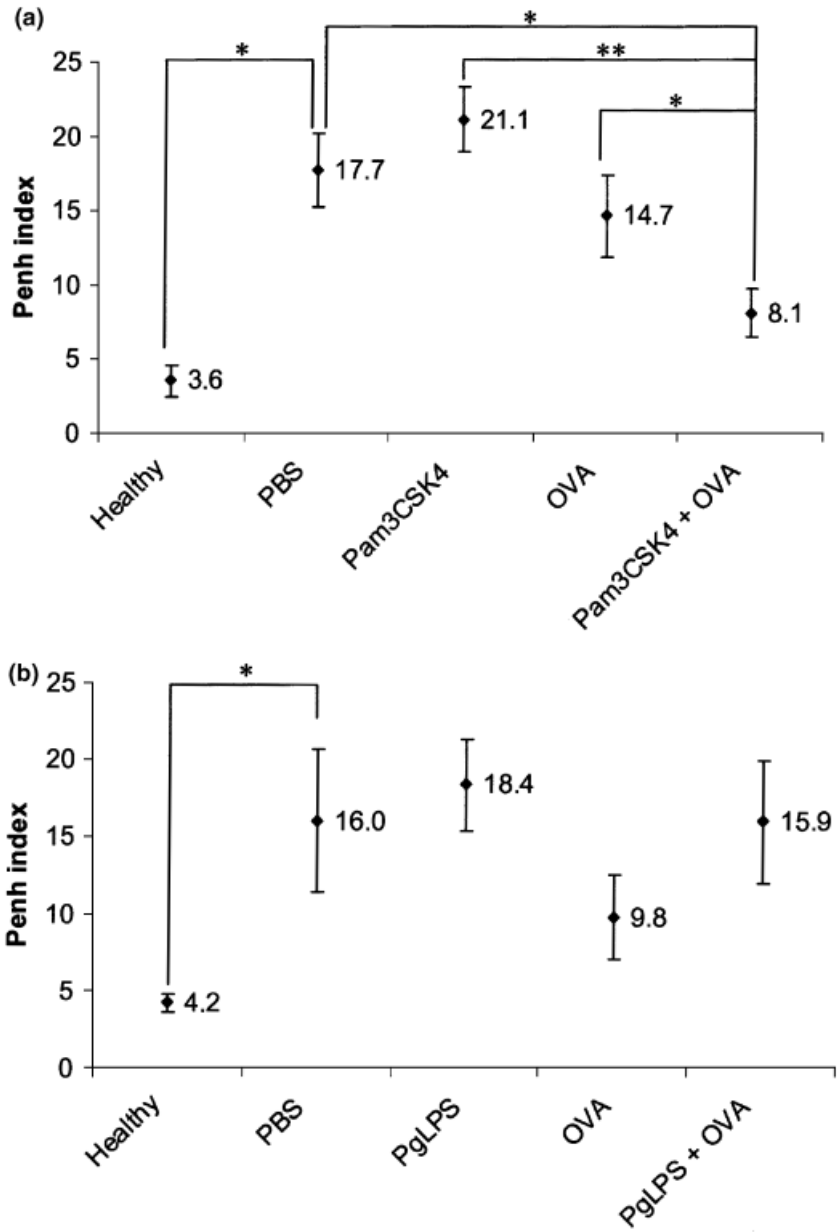

Fig. 5. Sublingual treatment with Pam3CSK4+OVA decreases airway hyperresponsiness and Th2 responses in OVA-sensitized mice. (a) OVA-sensitized mice were treated sublingually with either PBS, OVA (500 μg/dose), Pam3CSK4 (10 μg/dose) or OVA plus Pam3CSK4 (500 μg and 10 μg/dose, respectively) diluted in 50 μL PBS. AHR was evaluated by whole-body plethysmography and expressed as a Penh index as described in 'Material and methods'. Data shown (mean ± SEM) were obtained from eight mice per group. $^{*}P<0.05$; $^{**}P<0.02$. (b) OVA-sensitized mice were treated sublingually with PgLPS (10 μg/dose) as a TLR2 ligand, as described above. OVA, ovalbumin; PBS, phosphate-buffered saline; AHR, airway hyperresponsiveness; PgLPS, *Porphyromonas gingivalis* lipopolysaccharide; TLR, Toll-like receptor; Th2, T-helper type 2.

compared with that of PBS-treated animals, the AHR of mice treated sublingually with OVA alone is only marginally improved, with a mean Penh index decreasing from 21.1 to 14.7 in this experiment (Fig. 5a). OVA administered together with Pam3CSK4 significantly reduces AHR when compared with OVA alone ($P<0.05$), whereas Pam3CSK4 alone has no effect (Fig. 5a). As expected, no significant reduction of AHR is observed in OVA-sensitized mice receiving OVA plus PgLPS sublingually (Fig. 5b).

Immune mechanisms associated with Pam3CSK4-mediated induction of tolerance via the sublingual route

We first analysed immunological changes associated with tolerance induction in our SLIT model in BALB/c mice. Lung histology did not reveal any differences between the groups in terms of cellular infiltration or mucus secretion (data not shown). Also, we failed to detect significant changes in OVA-specific IgE or IgG antibodies in sera or IgA responses in the

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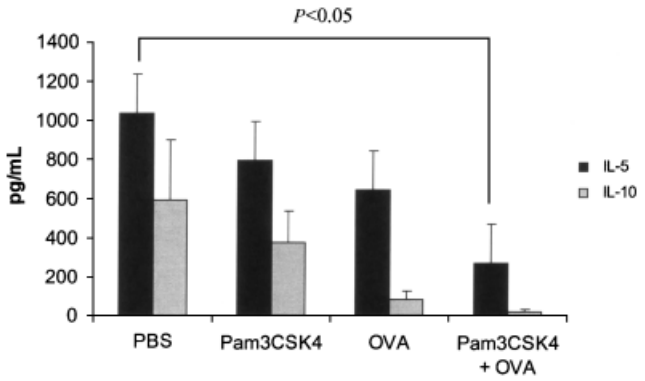

Fig. 6. Production of IL-5 and IL-10 in draining lymph nodes. At the end of the sublingual desensitization with either PBS, OVA (500 μg/dose), Pam3CSK4 (10 μg/dose) or OVA plus Pam3CSK4 (500 μg and 10 μg/dose, respectively), cells were harvested from superficial cervical lymph nodes recovered from each individual mouse. Cells were cultured for 3 days in the presence of OVA (25 μg/mL). IL-5 and IL-10 secretion was measured in culture supernatants by ELISA. Data shown are the mean ± SEM of results obtained in eight mice. OVA, ovalbumin; PBS, phosphate-buffered saline.

lungs from any of the treated animals (data not shown). To assess immunological changes locally, cervical lymph nodes were harvested after 2 months of SLIT and cultured in the presence of OVA for 3 days. As shown in Fig. 6, IL-5 and IL-10 production measured by ELISA in culture supernatants is substantially lower ($P<0.05$ for IL-5) in animals receiving Pam3CSK4 plus OVA, compared with Pam3CSK4 or OVA alone. The concomitant decrease of IL-5 and IL-10 production demonstrates that OVA-specific Th2 responses are down-regulated in these experiments [30]. No TGF-β or IFN-γ production was detected in draining lymph nodes (data not shown).

In a second set of experiments, we assessed the effect of Pam3CSK4 on cytokine gene expression by $CD11b^+$ $CD11c^-$ MHC class II^+ sublingual/oral DCs isolated by cell sorting from naïve BALB/c mice, as described in

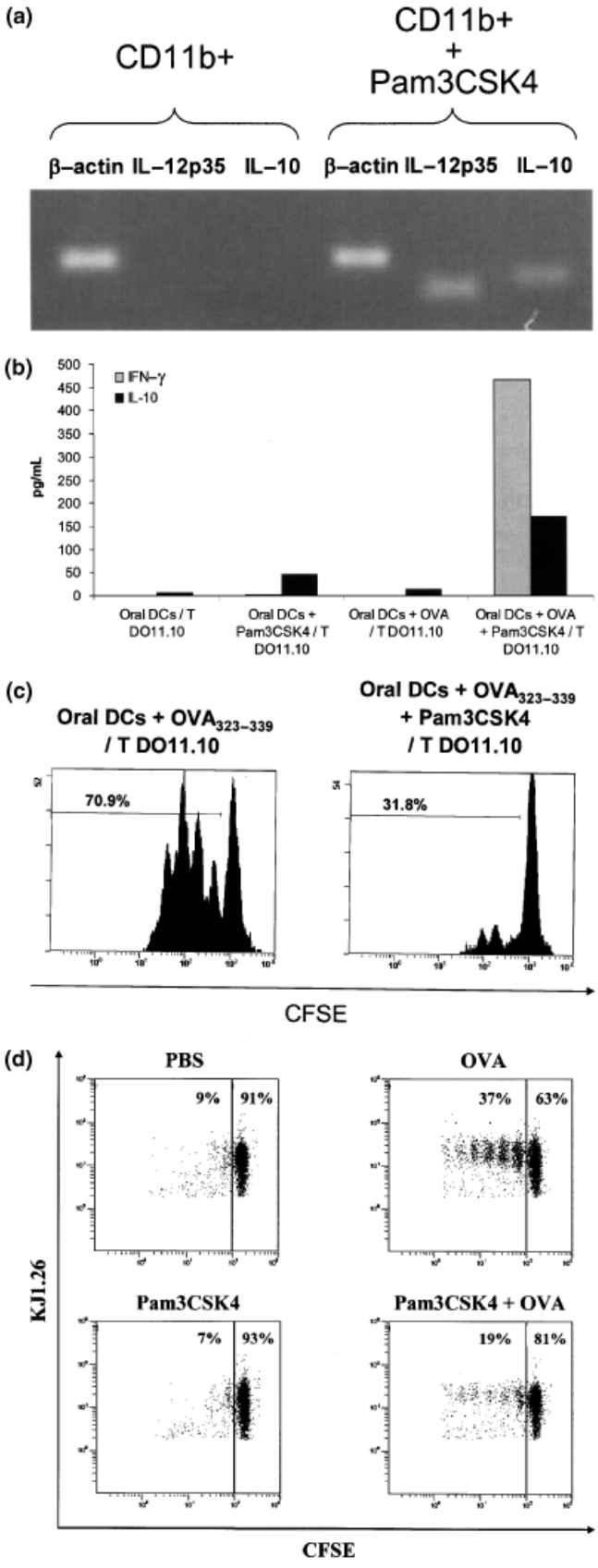

Fig. 7. Mechanisms associated with tolerance induction via the sublingual route. (a) $CD11b^+$ $CD11c^-$ MHC class II^+ oral DCs were isolated from sublingual/oral tissues using a cell sorter and subsequently cultured for 24 h in the presence of Pam3CSK4 (1 μg/mL) or medium. Total RNA was isolated and IL-10 and IL-12p35 gene expression was analysed by PCR. (b) $CD11b^+$ $CD11c^-$ MHC class II^+ oral-treated DCs were cultured with CFSE-labelled DO11.10 $CD4^+$ T cells for 3 days in the presence of 1 μg/mL $OVA_{323-339}$ or medium only as described in 'Material and methods'. IFN-γ and IL-10 production was measured in culture supernatants by CBA. (c) Proliferation in response to OVA was analysed by flow cytometry after staining of CFSE-labelled OVA-specific $CD4^+$ T cells with the anti-clonotypic antibody KJ1.26. No proliferation was detected in the absence of $OVA_{323-339}$. (d) 5×10^6 purified CFSE-labelled DO11.10 $CD4^+$ T cells were adoptively transferred into naïve BALB/c mice by a retroorbital intravenous injection. After 24 h, mice were treated via the sublingual route with either soluble PBS, OVA, Pam3CSK4 or Pam3CKS4 plus OVA as described in 'Material and methods'. At day 8, proliferation of transferred cells was assessed by flow cytometry using the anti-clonotypic antibody KJ1.26. Results shown are representative of two independent experiments. DCs, dendritic cells; CFSE, carboxyfluorescein diacetate succinimydil ester; CBA, cytometric bead array.

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'Material and methods'. These myeloid DCs represent the main subset of sublingual/oral APCs in mice and were confirmed to express high levels of TLR2 [31]. After treatment for 24 h with Pam3CSK4 (1 μg/mL), both IL-10 and IL-12p35 genes are induced in oral DCs when compared with cells cultured in the presence of medium alone (Fig. 7a). In the presence of $OVA_{323-339}$ (1 μg/mL), oral DCs treated by Pam3CSK4 trigger the production of high levels of both IFN-γ and IL-10 by naïve $CD4^+$ T cells (Fig. 7b). Under culture conditions with $OVA_{323-339}$ or Pam3CSK4 alone, no significant production of any of these cytokines is detected (Fig. 7b). In agreement with our results obtained with BMDC/T co-cultures, DO11.10 $CD4^+$ T cells cultured with Pam3CSK4-treated oral DCs exhibit a decreased proliferation in the presence of $OVA_{323-339}$ compared with $CD4^+$ T cells cultured with untreated oral DCs (Fig. 7c).

To further investigate the impact of sublingual administration of Pam3CSK4 on T cell responses *in vivo*, we performed adoptive transfer experiments to assess the proliferation of OVA-specific T cells in cervical lymph nodes of naïve BALB/c mice. To this end, CFSE-labelled OVA-specific $CD4^+$ T cells isolated from DO11.10 mice were adoptively transferred into healthy BALB/c mice. The next day, groups of four mice were immunized sublingually with a single dose of either PBS, OVA (500 μg), Pam3CSK4 (10 μg) or OVA plus Pam3CSK4 (500 μg and 10 μg, respectively) in 50 μL PBS. After 8 days, cervical lymph nodes were removed, cells were pooled and the proliferation of OVA-specific $CD4^+$-transferred T cells (detected with the anti-clonotypic antibody KJ1.26) was analysed by flow cytometry (Fig. 7d). In mice treated with PBS, only 9% of proliferating OVA-specific T cells are detected. Sublingual administration of OVA alone leads to the proliferation of 37% T cells. Interestingly, treatment with OVA plus Pam3CSK4 reduces T cell proliferation in cervical lymph nodes by approximately 50% when compared with OVA alone. These results confirm the local priming of OVA-specific T cells in draining lymph nodes after sublingual immunization and, further, that the TLR2 agonist Pam3CSK4 used as an adjuvant inhibits OVA-specific T cell proliferation *in vivo*.

Discussion

Allergen-specific immunotherapy remains the only efficient causal treatment of type I respiratory allergies [1–3]. Specific immunotherapy was first reported to induce a Th2 to Th1 shift in allergen-specific T cell responses [4–8]. Recent studies suggest that SCIT, and likely SLIT, also elicit allergen-specific Tregs, including both $CD4^+CD25^+$ Fox $p3^+$ and/or IL-10-secreting Tr1 cells [9–15]. These Tregs are known to mediate their immunomodulatory properties in part through the production of the anti-inflammatory cytokine IL-10 [9–15]. Thus, we reasoned that adjuvants capable of stimulating IFN-γ and IL-10 secretion by naïve $CD4^+$ T cells could improve the efficacy of allergy vaccines. Most particularly, given the growing interest in SLIT as a means to induce allergen-specific tolerance [20, 21], we focused on candidate adjuvants that could drive allergen-specific T cell responses towards Th1 and/or Tregs when applied on potentially tolerogenic DCs from the oral mucosa [33].

Various reports in the literature suggest that distinct TLR2 ligands can promote either Th1 [34], Th2 [35–37], Treg [38], Th1/Treg differentiation [39] or alternatively have no effect on T cell polarization [40, 41]. In our *in vitro* studies, among the three TLR2 ligands tested (Pam3CSK4, PgLPS and LTA), the synthetic lipopeptide Pam3CSK4 is the most powerful inducer of IL-12p40 and IL-10 secretion by murine BMDCs. In addition, we demonstrate a similar effect of Pam3CSK4 on purified $CD11b^+$ $CD11c^-$ MHC class II^+ oral myeloid DCs, i.e. the main population of oral APCs in mice [31]. As expected from these results, only Pam3CSK4-treated DCs support the differentiation of naïve $CD4^+$ T cells into IFN-γ (Th1)- and IL-10-producing T cells (potentially Tregs), this also leading to a decrease in T cell proliferation. Intracytoplasmic cytokine staining reveals the presence of two distinct subsets of IL-10-secreting T cells depending on whether they also produce IFN-γ or not. Collectively, our results establish that Pam3CSK4 has unique immunomodulatory properties among the TLR2 ligands. A noteworthy fact is that these results are in agreement with the observation that another synthetic lipopeptide (LP40) acting through TLR2 also stimulates IFN-γ and IL-10 secretion by naïve $CD4^+$ T cells and suppresses IL-4 production by Th2-commited T cells [39].

The capacity of Pam3CSK4 to enhance tolerance induction was further documented in an SLIT model of established asthma in BALB/c mice sensitized to OVA. Animals treated sublingually with the antigen associated with Pam3CSK4 exhibit a significantly lower AHR in comparison with the mice treated with OVA alone. The latter is correlated with a reduced production of Th2 cytokines in draining lymph nodes. Furthermore, when administered sublingually together with OVA, Pam3CSK4 decreases the proliferation of OVA-specific $CD4^+$ T cells in draining lymph nodes. Although we failed to detect the presence of Fox $p3^+$ T cells with a suppressive function, collectively, these data are consistent with the induction of OVA-specific Th1 and IL-10-producing Tr1 regulatory cells after sublingual treatement with OVA plus Pam3CSK4. In previous studies conducted in murine asthma models, Pam3CSK4 administered intraperitoneally was shown to improve established allergic AHR [34]. In contrast, Pam3CSK4 as well as peptidoglycans acting through TLR2 were rather found to promote Th2 responses in other studies [35–37]. The results obtained herein are consistent with our observation that other candidate adjuvants

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selected for their capacity to stimulate the production of IL-10 with or without IFN-γ by naïve $CD4^+$ T cells (e.g. *Lactobacillus plantarum*, dexamethasone plus 1,25-dihydroxyvitamin D3 or the synthetic LPS analogue OM-294-BA-MP) all enhance tolerance induction via the sublingual route in OVA-sensitized mice [30, 32]. Also in agreement with these results, TLR3 or TLR7/8 agonists eliciting the production of both IL-12 and IL-10 cytokines by immune cells were recently shown to prevent and suppress experimental asthma in mice [42]. Other cytokines, such as IL-35, which associates IL-12p35 with Epstein–Barr virus-induced gene 3 (Ebi3) [43], are also involved in tolerance induction. IL-35 is produced by Fox $p3^+$ Treg cells and contributes to their suppressive activity [43]. Further investigation is needed to assess the potential role of IL-35 in the induction of tolerance by Pam3CSK4.

Altogether, our findings suggest that the TLR2 agonist Pam3CSK4 exhibits unique immunomodulatory properties, characterized by a capacity to induce a strong IL-10 and IFN-γ production by naïve $CD4^+$ T lymphocytes. As such, it represents a good candidate adjuvant to enhance the efficacy of sublingual allergy vaccines.

Acknowledgements

The authors wish to thank Prof. David Klatzmann for his useful comments and suggestions on our experimental models. Vincent Lombardi is a recipient of a CIFRE fellowship from the French government.

Conflict of interest: Some of the authors are employees at the biopharmaceutical company Stallergènes SA.

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