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A synthetic triacylated pseudo-dipeptide molecule promotes Th1/TReg immune responses and enhances tolerance induction via the sublingual route

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Summary In this study, we tested two triacylated pseudo-dipeptidic molecules, OM-197-MP-AC and OM-294-BA-MP as candidate adjuvants for allergy vaccines. Both molecules induce human dendritic cell (h-DC) maturation and polarize naïve T cells toward the Th1 type with IFNγ production. Only OM-294-BA-MP induces IL10 gene expression both in monocyte-derived DCs and CD4+ naïve T cells. Sublingual administration of OM-294-BA-MP plus the antigen enhances tolerance induction in BALB/c mice with established asthma to ovalbumin with an impact on both airways hyperresponsiveness and lung inflammation. Given its Th1/Treg polarizing properties, OM-294-BA-MP is a valid candidate for sublingual allergy vaccines.
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Introduction

Allergen-specific immunotherapy is currently the only causal treatment for allergy. Subcutaneous immunotherapy (SCIT) has been considered as a reference therapy to treat allergies against insect venoms, house dust mites, grass and tree pollens or animal epithelia for almost a century [1–3]. However, the need for repeated injections over several years makes this treatment inconvenient, particularly for children. In addition, SCIT can be associated with severe, albeit infrequent side effects, including anaphylactic shocks [4]. In this context, sublingual immunotherapy (SLIT) has been developed 15 years ago as a safer and non-invasive alternative to the treatment of respiratory allergies [5–8].

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Recently, a meta-analysis based on 22 randomized clinical studies concluded that SLIT of allergies to either house dust mites, pollens (e.g. grass, parietaria, olive, ragweed, cupressus) or cat dander significantly reduces both symptoms (rhino-conjunctivitis, asthma), as well as the need for symptomatic medication in both adults and children [5—8]. The clinical efficacy of SLIT in humans was recently confirmed in a phase IIb/III randomized, double-blind placebo controlled trial involving more than 630 patients [9]. In this study, patients treated with grass pollen tablets exhibited a significant reduction in both symptoms (37%; $p < 0.0001$) and medication use (46%; $p < 0.02$). Given its well-established safety profile in humans, with more than a billion doses administered [6,10—13], sublingual immunotherapy is presently considered as a valid approach to induce antigen-specific tolerance in humans [14].

During SLIT, a natural allergen extract is placed under the tongue for at least 1—2 min prior to being swallowed. At the mucosal surface, allergens are captured by Langerhans-like dendritic cells [15], which subsequently migrate to draining cervical lymph nodes to present the antigen to naïve T cells [16,17]. Although allergic patients develop Th2 polarized allergen-specific CD4+ T cell responses characterized by IL4, IL5 and IL13 secretion [18,19], successful allergen-specific immunotherapy rather elicits Th1 responses [13,20—29], as well as IL10 producing T cells [30—33], likely regulatory T cells belonging to the CD4+CD25+ or Tr1 subsets.

Appropriate mucosal immunomodulators capable to induce antigen-specific Th1 and/or T regulatory immune responses are likely to enhance allergen-specific tolerance following sublingual immunization. In this context, we focus in the present study on two triacylated, non-toxic pseudo-dipeptidic analogues of lipid-A, named OM-197-MP-AC and OM-294-BA-MP as candidate adjuvants. Whereas the TLR4 agonist, OM-197-MP-AC is known to induce the maturation of human monocyte-derived DC and to increase ovalbumin-specific humoral and T helper cell responses [34,35], the immunomodulatory properties of OM-294-BA-MP remain to be assessed.

Herein, we demonstrate that OM-197-MP-AC induces IFNγ, whereas OM-294-BA-MP rather induces both IFNγ and IL10 gene expression by naïve human CD4+ T cells. OM-294-BA-MP, but not OM-197-MP-AC, significantly enhances SLIT efficacy in establishing tolerance in a therapeutic model of ovalbumin (OVA)-induced asthma in BALB/c mice.

Materials and method

Reagents

Recombinant human IL4 and human/mouse GM-CSF were obtained from Gentaur (Brussels, Belgium). Phosphate-buffer saline (PBS) and RPMI 1640 were purchased from Invitrogen (Carlsbad, CA). Alum was obtained from Pierce (Rockford, IL). LPS from *Escherichia coli* 0128:B12 and OVA grade V were purchased from Sigma (St. Louis, MO). For desensitization purposes, a purification step was added in order to remove contaminant endotoxin (LPS) from OVA. To this aim, the OVA protein was diluted in PBS at 20 mg/ml and filtered with a 0.45 μm filter (Milex SLHV 025 NS, Millipore). A 16/20 XK column (GE Health Care) was filled with 200 ml endotoxin removing gel (20339, Pierce), then washed with 3 column volumes of apyrogen water, 1% sodium deoxycholate (D5670, Sigma) and water. The column was equilibrated with 3 volumes of PBS, prior to loading the filtered OVA solution for 1 h to allow endotoxin binding and eluting OVA with PBS. Endotoxin content of such detoxified OVA was determined by Endochrom-K assay (R1708K, Charles River, Wilmington, MA) and always found to be less than 0.1 EU/μg of protein. Protein concentrations were determined using a spectrophotometer (Secomam, Alès, France), measuring absorption at 278 nm using a molecular extinction coefficient of 141 $mM^{-1}\,cm^{-1}$. The lactic acid bacterium, *Lactobacillus plantarum* was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA; No. 8014). OM-197-MP-AC (MP-AC stands for MonoPhosphate AminoCaproyl) and OM-294-BA-MP (BA-MP stands for Butyric Acid MonoPhosphate) are synthetic triacylated pseudo-dipeptidic molecules, [34] from OM-Pharma (Genève, Switzerland). For *in vitro* assays, adjuvants were used at 1—20 μg/ml as indicated in figure legends.

Adjuvant testing in human immune cells

Human-DCs (h-DCs) were generated from peripheral blood mononuclear cells (PBMCs) as previously described, with minor modifications [36]. Briefly, PBMCs were isolated from healthy volunteers by centrifugation of heparinized blood over a Ficoll-Paque plus gradient (Amersham Biosciences Piscataway, NJ). Following resuspension in culture medium (RPMI 1640 supplemented with 2 mM L-glutamine, 20 μg/ml gentamicin, 50 μM 2-mercaptoethanol, 1% nonessential amino acids (all from Invitrogen), and 10% fetal bovine serum (Gentaur)), cells were allowed to adhere to 75 ml Falcon flasks. After 2 h at 37 °C, nonadherent cells were removed and adherent cells were recovered and cultured for 7 days in 20 ml medium containing GM-CSF (80 ng/ml) and IL4 (25 ng/ml). Nonadherent cells corresponding to the h-DC-enriched fraction were harvested, washed, and used for subsequent experiments. Human-DC purity assessed by morphology and flow cytometry analysis (based on CD1a expression) was always superior to 90%. Allogeneic CD4+ naïve T lymphocytes were purified from PBMCs of healthy volunteers by magnetic bead separations (Invitrogen) using a mix of antibodies against CD14, CD16, CD56, CDw123, CD36, CD8, HLA Class II DR/DP, glycophorin A (all from Invitrogen) and CD45RO molecules (BD Biosciences, San Jose, CA). The purity of naïve CD45 RA+ T cells analyzed by FACS was always higher than 90%. To investigate the effect of candidate adjuvants on DC maturation and cytokine production, 5×10^5 h-DCs were incubated in 24-well plates in presence of either medium, adjuvants (20 μg/ml) or LPS (1 μg/ml). After 24 h, h-DCs were washed, and tested in terms of viability and surface phenotype. Supernatants were harvested and cytokine levels were determined using the cytokine beads array (CBA) technology. To assess an effect on T cell polarization, 10^4 h-DCs/well were further cultured in triplicate in presence of allogeneic CD4+ naïve T cells (10^5 cells/well) in 96-well flat-bottom microtiter

plates under a final volume of 200 μl. Cells and culture supernatants were collected after 4 h, 1 or 5 days of culture.

Adjuvant testing in murine immune cells

Bone marrow-derived dendritic cells (BM-DCs) were obtained as previously described [37]. Briefly, bone marrow cells (2×10^5 cells per ml) were incubated in RPMI 1640, supplemented with 10% foetal calf serum, antibiotics, 5×10^{-5} M beta-2-mercaptoethanol (all from Invitrogen) and 10 ng/ml GM-CSF. On day 7, BM-DCs were harvested using 10 ml of PBS/EDTA 5 mM for 5 min. CD4+ T cells from DO11.10 mice [38], transgenic for the T cell receptor specific for the OVA 323—339 epitope restricted to the H2d haplotype, were purified from spleens by magnetic bead separation using the mouse CD4 negative isolation kit (Invitrogen) according to the manufacturer's instructions. The resulting DO11.10 T cell preparations contained routinely 95—99% pure CD4+ T cells as confirmed by FACS analysis. For proliferation assays, DO11.10 T cells were labelled with 1 μM carboxy-fluorescein diacetate, succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) for 5 min at 37 °C in PBS and washed twice.

To assess the effect of candidate adjuvants on BM-DC maturation and cytokine production, 10^6 BM-DCs were incubated in presence of either complete medium, adjuvant (10 μg/ml) or LPS (2 μg/ml). After 24 h, BM-DCs were washed, and tested in terms of viability and surface phenotype. Supernatants were recovered and cytokine production was determined by ELISA. For *in vitro* antigen presentation assays, BM-DCs (5×10^4) were incubated with OVA (25 μg/ml) for 4 h, washed and co-cultured with 5×10^5 freshly isolated DO11.10 T cells in 200 μl complete medium. Supernatants were recovered at various time points (i.e. 4 h, 1, or 3 days) during the culture in order to perform cytokine secretion assays. In parallel, cells were harvested and lysed for mRNA isolation and quantitative PCR analysis (see Section RNA isolation and real-time PCR analysis). For *in vitro* proliferation assays, CFSE labelled DO11.10 CD4+ T cells were stimulated with BM-DCs and OVA (10 μg/ml) for 3 days. Cells were stained with the PE-KJ1.26 anti clonotypic mAb (BD Biosciences, San Jose, CA) directed to the TCR expressed on OVA-specific T cells from DO11.10 mice [38]. Proliferating T cells were detected by FACS on the basis of decreased CFSE-associated fluorescence.

FACS analysis of surface phenotype and cytokine production

For immunofluorescence staining of h-DCs, cells were harvested, washed in PBS and incubated for 20 min at room temperature with the following mAbs: fluorescein isothiocyanate (FITC) labelled anti-CD80 IgG1, or anti-HLA-DR IgG1 phycoerythrin (PE) labelled anti-CD86 IgG2b or anti-CD40 IgG1, phycoerythrin-cyanin 5.1 (PC5) labelled anti-CD83 IgG2b (all from Beckman Coulter, Villepinte, France). For immunofluorescence staining of murine BM-DCs, cells were harvested, washed in PBS and incubated for 20 min at room temperature with the following mAbs: PE-labelled anti-CD11c IgG, allophycocyanin (APC) labelled anti-MHC Class II (I-A/I-E) IgG2b or anti-CD40 IgG, anti-CD80 IgG or anti-CD86 IgG2a (all from eBiosciences). As a control, h-DCs and BM-DCs were stained with corresponding isotype-matched mAbs. Samples were analyzed using a FC500 flow cytometer (Beckman Coulter). Results were expressed as mean fluorescence intensity.

For cytokine production measurement, IL1α, IL6, IL10, IL12p70 and TNFα were analyzed in culture supernatants by flow cytometry using the human or mouse inflammation and Th1/Th2 CBA kit from BD Biosciences (San Jose, CA), as appropriate, according to the manufacturer's instructions.

RNA isolation and real-time PCR analysis

Total RNA was isolated from 10^6 human or murine T cells (RNeasy Mini kit, Qiagen) and cDNAs were synthesized using random hexamers (Taqman Reverse Transcription Reagents, Applied Biosystems, Foster city, CA), according to the manufacturer's protocols. PCR probes (Applied Biosystems) were selected from a gene bank from Applied custom assays and synthesized by Applied Biosystems, in order to amplify Tbet, GATA3, Foxp3, IFNγ, IL4, IL10 and TGFβ human or murine genes, respectively. Real-time PCR was performed with Applied Biosystems Predesigned TaqMan Gene Expression Assays and reagents, according to the manufacturer's specifications, using a 7300 Real-time PCR system (Applied Biosystems). PCR was performed for 30—40 cycles under the following conditions: 95 °C, 30 s; 55 °C, 30 s; 72 °C, 30 s. The level of transcription for each gene was normalized in comparison with the housekeeping β-actin gene.

Sublingual immunotherapy in BALB/c mice

Six- to eight-weeks-old BALB/c female mice were obtained from Charles River (L'Arbresle, France). For sensitization (Fig. 1), mice were immunized intraperitoneally (i.p.) on days 0 and 14 with 10 μg OVA adsorbed on 2 mg $Al(OH)_3$, administered in 100 μl PBS. At day 21, a 20 min aerosol challenge was performed with 1% (w/v) OVA on 4 consecutive days using an aerosol delivery system (Buxco Europe; Ltd., Winchester, UK). For tolerance induction (Fig. 1), 500 μg OVA with or without 5 μg of each adjuvant were applied sublingually to groups of eight mice twice a week during 2 months in 20 μl of PBS. Control mice were sensitized with OVA followed by sham tolerization with PBS or adjuvant alone. Measurements of airway hyperresponsiveness (AHR) were performed by whole body plethysmography (Buxco Europe; Ltd., Winchester, UK) and results were expressed as enhanced pause (Penh). The Penh index, expressed as an increase relative to the baseline airway resistance, was obtained by dividing the Penh value measured after exposure to 50 mg/ml inhaled metacholine with the one measured after inhalation of nebulized PBS, as previously described [39].

For lung histology, mice were killed by cervical dislocation, lungs were recovered and fixed in phosphate buffered formalin-zinc and embedded in paraffin wax. Sections were stained with hematoxylin, eosin and safran (HES) for the determination of cellular infiltrates.

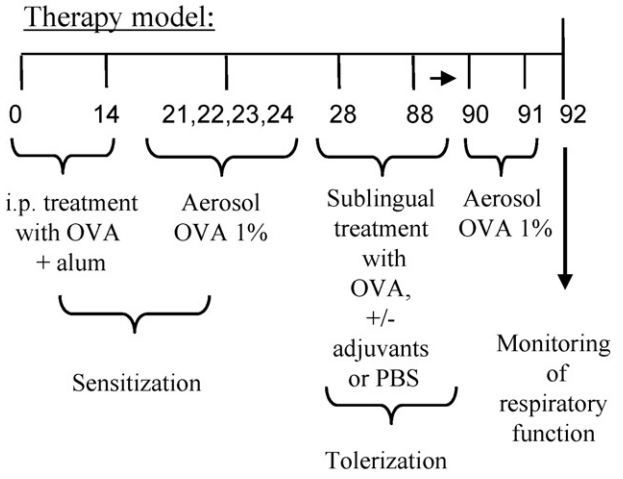


Figure 1 Experimental design. Mice were sensitized by intraperitoneal (i.p.) injections with OVA/Alum (days 0 and 14) followed by an aerosol challenge (days 21—24) with OVA. BALB/c mice were sublingually treated twice a week with either PBS, OM-197-MP-AC (5 μg/dose) or OM-294-BA-MP (5 μg/dose), with or without OVA (500 μg/dose) (days 28—88). After an aerosol challenge (days 90—91) with OVA, AHR measurements were performed by whole body plethysmography at day 92.

For *in vivo* proliferation assay, CFSE labelled purified CD4+ T cells (5×10^6) cells were adoptively transferred by retroorbital intravenous injections into BALB/c mice at day 0. After 24 h, mice were sublingually treated with either PBS, OM-197-MP-AC (5 μg), OM-294-BA-MP (5 μg) or *L. plantarum* (2×10^8 of live bacteria) with or without OVA (500 μg) in 20 μl. At day 8, cervical LNs were harvested, OVA-specific T cells were stained with the anticlonotypic PE-KJ1.26 mAb and the percentage of proliferating T cells was evaluated by FACS based on a decrease in CFSE-associated fluorescence.

Statistical analysis

Penh or mean fluorescence intensity values were plotted using the Prism software (GraphPad Software, Inc., CA) and compared using the student's *t*-test. The limit of statistical significance considered was $p < 0.05$.

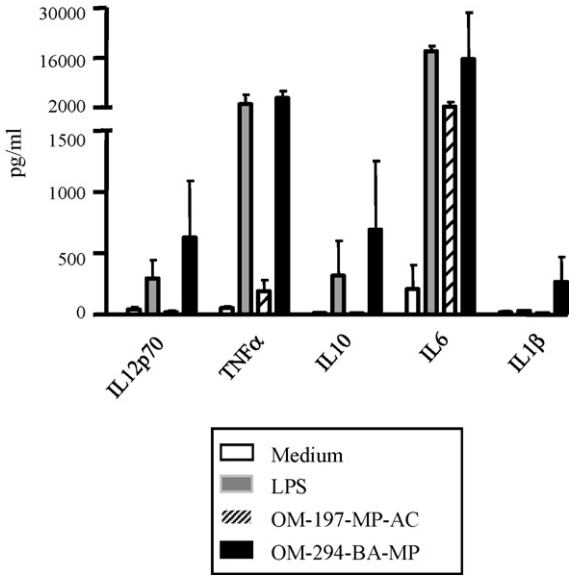


Figure 2 Cytokine production by human DCs following treatment with OM-197-MP-AC or OM-294-BA-MP. Monocytes-derived immature DCs were cultured for 24 h with either medium alone (white bar), LPS (1 μg/ml, grey bar), OM-197-MP-AC (20 μg/ml, hatched bar) or OM-294-BA-MP (20 μg/ml, black bar). Supernatants were tested for the presence of IL12p70, TNFα, IL10, IL6 and IL1β by cytometric bead array assay. Data are shown as mean concentrations ± S.D. obtained from three independent experiments.

Results

OM-197-MP-AC and OM-294-BA-MP induce human and murine dendritic cell maturation

We first investigated the capacity of synthetic triacylated lipid A molecules OM-197-MP-AC and OM-294-BA-MP [34] to induce maturation as well as cytokine production in DCs. Human DCs cultured in medium alone or in presence of LPS were used as negative and positive control, respectively. Incubation of h-DCs with 20 μg/ml OM-197-MP-AC or OM-294-BA-MP up-regulated surface expression of both CD83, CD40, CD80 and CD86 molecules to a level comparable to 1 μg/ml LPS (Table 1). OM-197-MP-AC also induced TNFα as well as IL6 secretion (Fig. 2). OM-294-BA-MP strongly stim-

Table 1 Human dendritic cell (h-DC) maturation induced by OM-197-MP-AC or OM-294-BA-MP

	HLA-DR	CD83	CD40	CD80	CD86
h-DC alone	239.21 ± 50.40	7.04 ± 2.13	1060 ± 125.46	54.68 ± 13.13	80.63 ± 45.73
h-DC + LPS	219.55 ± 75.22 (ns)	44.93 ± 8.00**	3091.23 ± 695.62**	141.55 ± 8.45***	706.89 ± 29.97***
h-DC + OM-197-MP-AC	268.21 ± 83.47 (ns)	26.89 ± 2.89**	3001.23 ± 802.68*	101.38 ± 12.14*	451.89 ± 103.64**
h-DC + OM-294-BA-MP	245.55 ± 63.32 (ns)	29.73 ± 7.39**	2967.89 ± 659.05**	113.21 ± 9.1**	632.89 ± 87.74***

Monocyte-derived immature dendritic cells were obtained from PBMCs of healthy donors ($n = 3$). Cells were incubated with adjuvants (20 μg/ml) or LPS (2 μg/ml) for 24 h and the expression of various surface molecules was assessed by indirect immunofluorescence using specific antibodies and flow cytometry. Results are expressed as mean fluorescence intensity ± S.D. Mean fluorescence intensity values were compared using Student's *t*-tests. Not significant (ns), $^{*}p < 0.02$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared with the mean fluorescence intensity of h-DC alone.

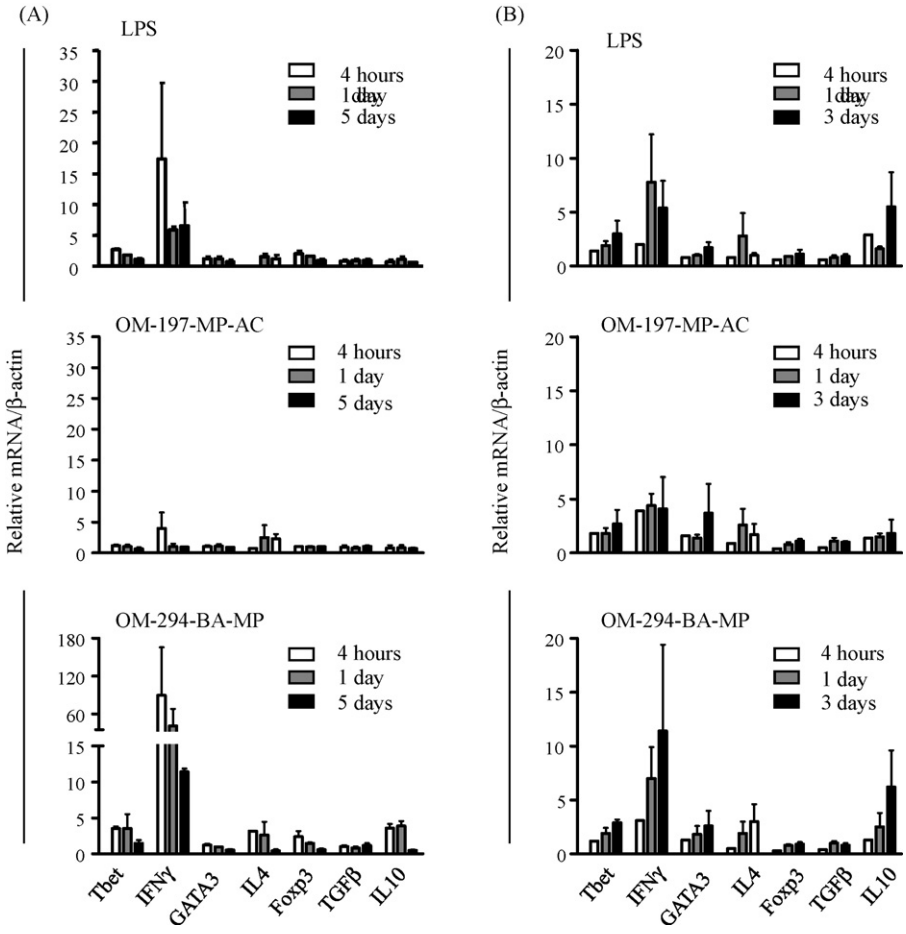


Figure 3 Gene expression in T cells co-cultured with human or murine DCs pre-treated by OM-197-MP-AC or OM-294-BA-MP. (A) Naïve CD4+ T lymphocytes were co-cultured for 4 h (white bar), 1 day (grey bar) or 5 days (black bar) with allogeneic h-DCs pre-treated with either medium alone, LPS (1 μg/ml), OM-197-MP-AC (20 μg/ml) or OM-294-BA-MP (20 μg/ml). Gene expression was analyzed by quantitative PCR. Data are expressed as relative amounts of mRNA in T cells co-cultured with h-DC pre-treated with LPS (upper panel), OM-197-MP-AC (center panel) or OM-294-BA-MP (lower panel) in comparison with T cells incubated with h-DC alone. Data are normalized to amounts of β-actin, and shown as the mean ± S.D. of three independent experiments. (B) OVA-specific CD4+ T lymphocytes from DO11.10 mice were co-cultured for 4 h (white bar), 1 day (grey bar) or 3 days (black bar) with murine BM-DCs that were pre-treated with either medium alone, LPS (2 μg/ml), OM-197-MP-AC (10 μg/ml) or OM-294-BA-MP (10 μg/ml) in presence of OVA (10 μg/ml). Gene expression was analyzed by quantitative PCR. Data are expressed as relative amounts of mRNA in T cells co-cultured with BM-DCs pre-treated with LPS (upper panel), OM-197-MP-AC (center panel) or OM-294-BA-MP (lower panel) in comparison with T cells incubated with BM-DCs alone. Data are normalized to amounts of β-actin and are shown as the mean ± S.D. of three independent experiments.

ulated both IL12p70, TNFα, IL10, IL6 and IL1β production by h-DCs (Fig. 2). Both OM-197-MP-AC and OM-294-BA-MP induced murine BM-DC maturation, with up-regulation of MHC-II, CD40, CD80 and CD86 surface expression (data not shown).

Effect of OM-197-MP-AC and OM-294-BA-MP on CD4+ T cell polarization

We next analyzed the polarization of allogeneic naïve CD4+ T cells incubated with adjuvant-treated h-DCs. To this aim, h-DCs were incubated for 24 h with either OM-197-MP-AC (20 μg/ml), OM-294-BA-MP (20 μg/ml) or LPS (1 μg/ml). Non-activated h-DCs were used as a control. Human DCs were washed and incubated with allogeneic naïve CD4+ T cells. The expression of genes encoding transcription factors or cytokines was subsequently monitored after either 4 h, 1 or 5 days by quantitative RT-PCR. As expected, LPS induces IFNγ gene expression in naïve T cells as early as after 4 h of culture (Fig. 3A, upper panel). OM-197-MP-AC only slightly up-regulates IFNγ gene expression, after 4 h (Fig. 3A, center panel). In contrast, OM-294-BA-MP markedly increases IFNγ gene expression by at least 80-fold when compared with T cells incubated with h-DC alone, and this up-regulation is maintained throughout the 5-day monitoring period (Fig. 3A, lower panel). Interestingly, OM-294-BA-MP also induces within 24 h a 5-fold increase in IL10 mRNA (Fig. 3A, lower panel).

These results were also confirmed in murine T cells. To this aim, murine BM-DCs were incubated with either OM-197-MP-AC (10 μg/ml) or OM-294-BA-MP (10 μg/ml) for 24 h, then

washed and co-cultured with OVA-specific CD4+ T cells from DO11.10 transgenic mice. Non-activated or LPS (2 μg/ml) treated BM-DCs served as negative and positive controls, respectively. Fig. 3B shows that both OM-197-MP-AC (center panel) and OM-294-BA-MP (lower panel) were able to stimulate IFNγ gene expression. IL10 gene expression was only found in T cells co-cultured with OM-294-BA-MP treated BM-DCs (Fig. 3B, lower panel). Taken together, these results obtained in both humans and murine immune cells, indicate that OM-294-BA-MP induces a Th1/Treg CD4+ T cell response whereas OM-197-MP-AC rather elicits a Th1 pattern of differentiation.

We further tested OM-197-MP-AC and OM-294-BA-MP for their capacity to modulate T cell stimulation by DCs. Murine BM-DCs were incubated with either medium, LPS (1 μg/ml), OM-197-MP-AC (10 μg/ml) or OM-294-BA-MP (10 μg/ml). After 24 h, cells were washed and co-cultured for 3 days with CFSE-labelled DO11.10 CD4+ T cells with or without detoxified OVA (10 μg/ml). OVA-specific T cell proliferation was barely detectable in all groups when OVA was omitted (Fig. 4). BM-DCs co-cultured with T cells in the presence of OVA showed a moderate proliferation (with only 25% proliferating cells, Fig. 4). In contrast, BM-DCs activated with either LPS, OM-197-MP-AC or OM-294-BA-MP increased T cell proliferation significantly (Fig. 4).

OM-294-BA-MP, but not OM-197-MP-AC, improves clinical efficacy in a murine model of sublingual immunotherapy

As described elsewhere [40], mice sensitized with OVA using the protocol summarized in Fig. 1 develop airways hyper responsiveness associated with elevated Penh values detectable by whole body plethysmography, as well as signs of lung inflammation with cellular infiltrates. OM-197-MP-AC and OM-294-BA-MP were tested as candidate adjuvants for immunotherapy in this *in vivo* murine model of established asthma. OM-197-MP-AC (5 μg per dose) and OM-294-BA-MP (5 μg per dose) were administered sublingually with or without OVA (500 μg per dose) in a total volume of 20 μl, in order to test their capacity to enhance the efficacy of allergen-specific immunotherapy in lowering AHR. Healthy mice used as a control (i.e. non-sensitized) in those experiments exhibit, as expected, low Penh values (Fig. 5), whereas OVA-sensitized animals treated sublingually with PBS alone display a high AHR. Sublingual treatment with soluble OVA alone twice a week for two months has a very moderate impact on AHR (Fig. 5). In contrast, sublingual treatment with OVA administered together with OM-294-BA-MP, but not OM-197-MP-AC, induces a statistically significant ($p < 0.05$) reduction of AHR in most animals

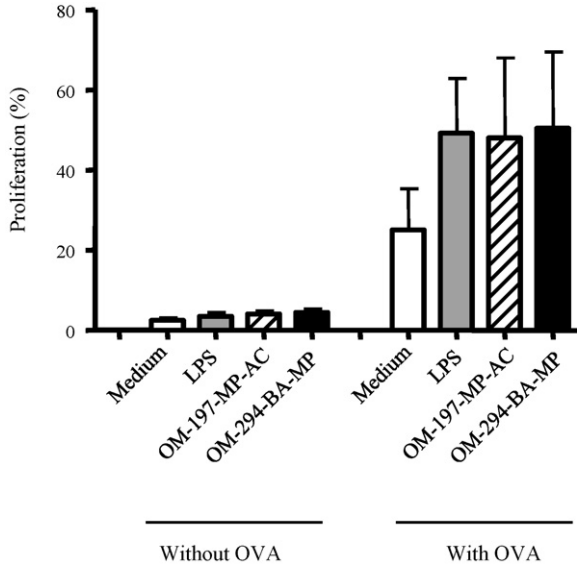


Figure 4 OM-197-MP-AC or OM-294-BA-MP pre-treatment of BM-DCs enhances T cell proliferation. OVA-specific CD4+ T lymphocytes from DO11.10 mice were labelled with 0.5 μM CFSE and co-cultured with BM-DCs pre-treated with either medium alone, LPS (2 μg/ml), OM-197-MP-AC (10 μg/ml) or OM-294-BA-MP (10 μg/ml) with or without OVA (10 μg/ml). After 3 days, cells were harvested, stained with PE-anti TCR (clone KJ1-26) mAbs. T cell proliferation was evaluated by FACS based on a decrease in CFSE-associated fluorescence using a FC500 flow cytometer. Data are shown as the mean ± S.D. of three independent experiments.

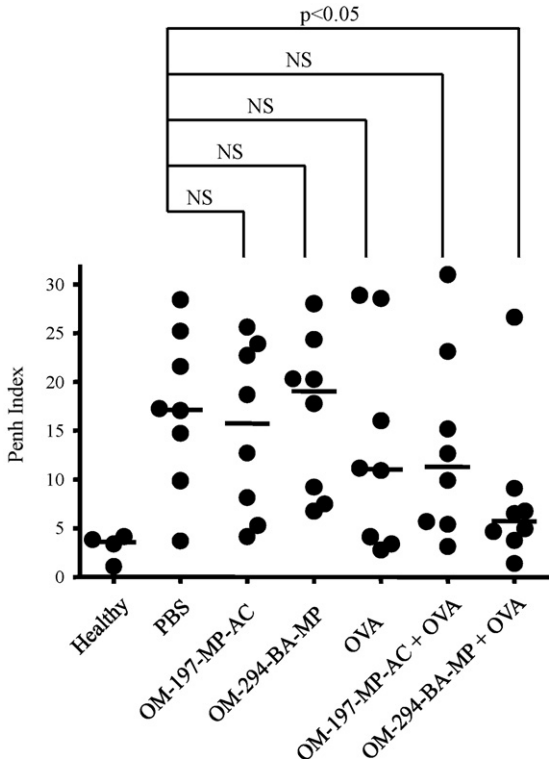


Figure 5 OM-294-BA-MP enhances tolerance induction via the sublingual route. Airway responsiveness was determined by measuring the Penh value in response to metacholine (50 mg/ml) as described in methods. Between four to eight individual mice were analyzed within each group. Horizontal bars represent the median response within each group with each dot representing the Penh value obtained in a single animal. Results are representative of two independent experiments.

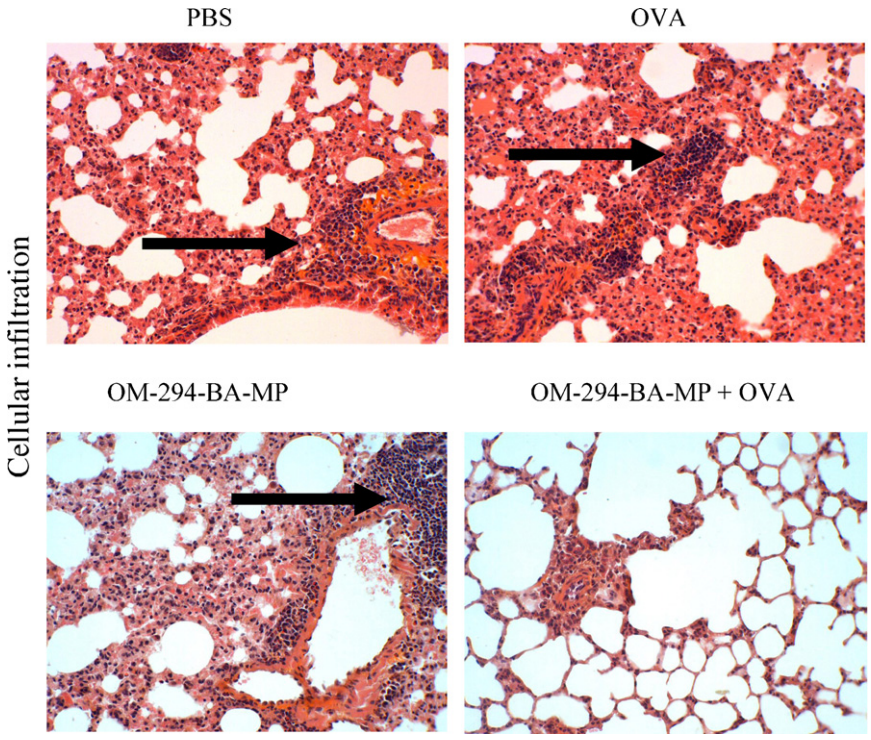


Figure 6 Therapeutic SLIT with OM-294-BA-MP + OVA reduces bronchial inflammation. Representative paraffin lung sections stained with HES are shown. Samples obtained from mice treated with either PBS, soluble OVA, OM-294-BA-MP alone or OM-294-BA-MP + OVA are shown (100-fold magnification). Arrows indicate cellular infiltrates.

when compared with mice treated with PBS (Fig. 5). Importantly, neither OM-197-MP-AC nor OM-294-BA-MP alone improves AHR, suggesting that antigen-specific immune mechanisms are involved in tolerance induction in this model.

The evaluation of bronchial inflammation by lung histology in those animals confirmed that sublingual treatment with OM-294-BA-MP + OVA leads to a reduction of lung inflammation, with less cellular infiltrates detectable in tissue sections (Fig. 6 and Table 2). Importantly, neither PBS, OM-294-BA-MP, nor OVA has any effect on lung inflammation (Fig. 6 and Table 2). Treatment with OM-197-MP-AC as an adjuvant had no effect on established lung inflammation (data not shown).

Assessment of immune responses after sublingual administration of OM-197-MP-AC or OM-294-BA-MP

Whereas sensitized BALB/c mice exhibit OVA-specific Th2 responses [40], sublingually treated mice with OVA plus OM-197-MP-AC or OM-294-BA-MP did not exhibit any detectable changes in OVA-specific IgE, IgG1 or IgG2a seric titers (data not shown). We also failed to detect by Elispot any significant changes in the number of OVA-specific IL5 or IL10 producing T cells in the spleen (data not shown).

To evaluate whether adjuvant molecules promote or inhibit CD4+ T cell priming within draining cervical lymph node after sublingual administration [40,41], we performed adoptive transfer experiments. To this aim, CFSE labelled

Table 2 Perivascular and alveolar inflammation analysis in OVA-sensitized mice treated sublingually with PBS, OVA, OM-294-BA-MP or OM-294-BA-MP + OVA

Groups	*n*	Perivascular inflammation			Alveolar inflammation			
		+	++	+++	+	++	+++	++++
PBS	7			7/7			2/7	5/7
OVA	8		4/8	4/8		1/8	3/8	4/8
OM-294-BA-MP	8			8/8		1/8	4/8	3/8
OM-294-BA-MP + OVA	8	1/8	5/8	2/8	2/8	4/8	1/8	1/8

BALB/c mice (7—8 per group) were sensitized to OVA as described in Fig. 1. OVA-sensitized mice were sublingually treated twice a week with either PBS, or OM-294-BA-MP (5 μg/dose), with or without OVA (500 μg/dose) (days 28—88). After an aerosol challenge (both on days 90 and 91) with 1% (w/v) OVA, lungs were removed and a semi-quantitative analysis of inflammation was performed with a blind assessment of the presence of cellular infiltrates (i.e. lymphoytes, plasmocytes and eosinophils). Data are expressed as numbers of animals exhibiting signs of inflammation ranked from low (+), moderate (++), important (+++), to severe (++++).

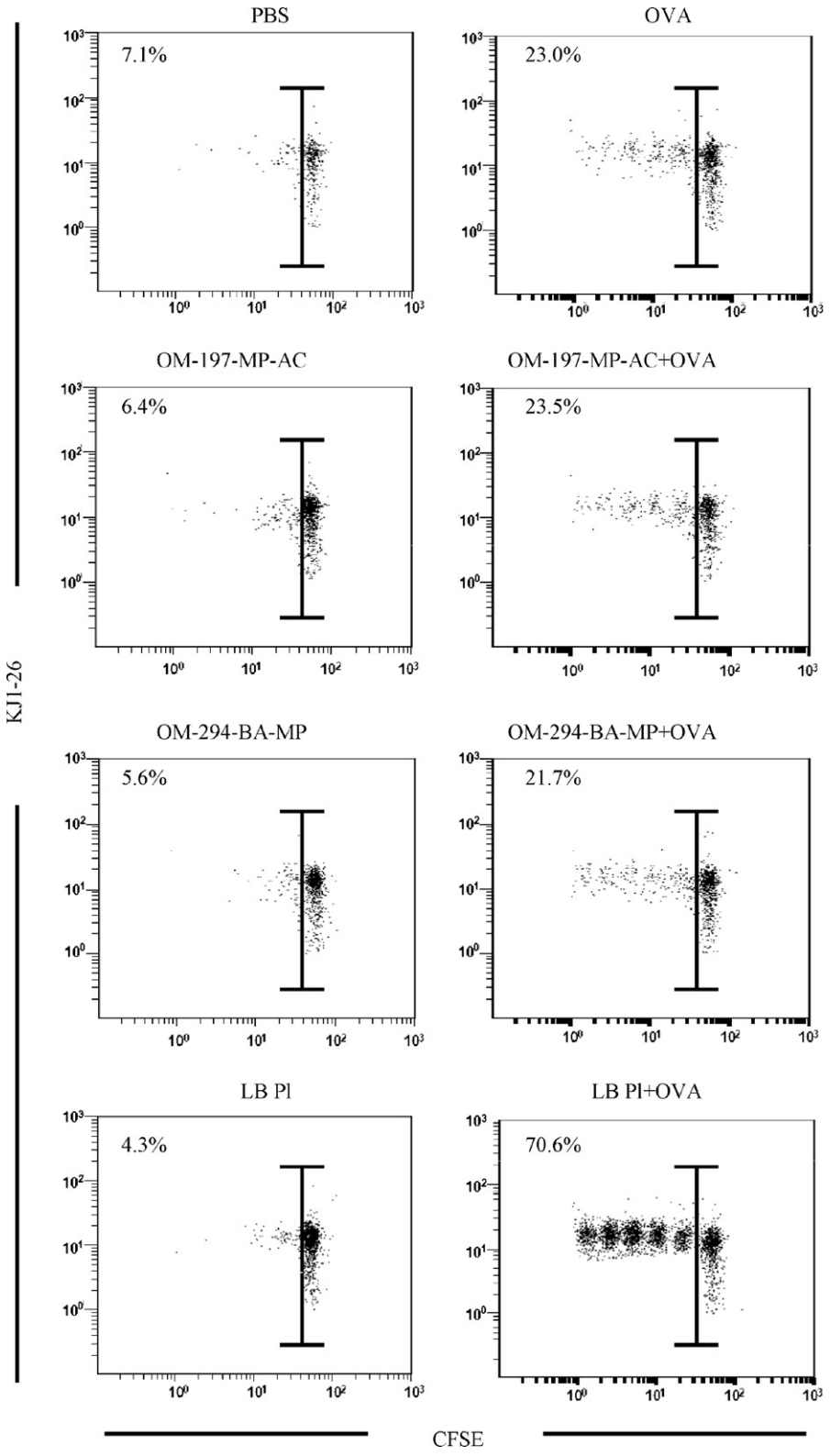


Figure 7 T cell priming in cervical lymph nodes in sublingually treated mice. Purified DO11.10 CD4+ T cells (5×10^6) were adoptively transferred by intravenous (i.v.) injection into BALB/c mice at day 0. Twenty-four hours later, mice were treated by the sublingual route with either soluble OVA (500 μg), OM-197-MP-AC (5 μg) plus OVA (500 μg) or OM-294-BA-MP (5 μg) plus OVA (500 μg) as described in methods. PBS, OM-197-MP-AC (5 μg) and OM-294-BA-MP (5 μg) treatments were used as negative controls. At day 8, cervical LNs were removed. OVA-specific T cells were stained with the PE-KJ1.26 mAb and the percentage of proliferating T cells was evaluated by FACS based on a decrease in CFSE-associated fluorescence. Data are representative of three independent experiments.

DO11.10 CD4+ T cells (5×10^6) were injected intravenously into BALB/c mice at day 0. After 24 h, mice were sublingually treated with either PBS, OM-197-MP-AC, OM-294-BA-MP or *L. plantarum* (LB-Pl) as a control [41] with or without OVA. At day 8, cervical LNs were harvested and *in vivo* OVA-specific T cell proliferative responses were analyzed by FACS. T cell proliferation was not detectable in cervical LNs in PBS, OM-197-MP-AC, OM-294-BA-MP or LB-Pl treated mice with only 7.1, 6.4, 5.6 and 4.3% proliferating T cells, respectively. In contrast, OVA, OM-197-MP-AC plus OVA or OM-294-BA-MP plus OVA treatments similarly induced a moderate proliferation (23.0, 23.5 and 21.7%, respectively, Fig. 7) in cervical LN cells. As previously described [41] LB-Pl + OVA strongly increased T cell proliferation up to 70%. These results confirm a local priming of OVA-specific T cells following sublingual immunization under those conditions, without any further detectable impact of OM-197-MP-AC or OM-294-BA-MP on proliferation when co-administrated with the antigen.

We also analyzed by FACS the impact of sublingual administration of OM-294-BA-MP plus OVA on Foxp3 expression in CFSE labelled DO11.10 CD4+ T cells adoptively transferred into BALB/c mice in cervical lymph nodes. We did not detect any increase in Foxp3 expression in both proliferating and non-proliferating T cells (data not shown).

Discussion

The sublingual route of administration is now established as a safe and efficacious route to induce allergen-specific tolerance in humans [5—14]. Several studies suggest that allergen-specific immunotherapy redirects allergen-specific CD4+ T cell responses from a Th2 to a Th1 response [13]. Also successful allergen immunotherapy appears to be associated with the induction of IL10-producing regulatory T-cells including both Foxp3 + CD4 + CD25+ and Tr1 cells [30—33]. In this context, adjuvants promoting IFNγ and/or IL10 production by naïve T cells are expected to enhance allergen-specific tolerance induction in the course of immunotherapy.

Triggering toll like receptor (TLR) with lipid A derivatives is known to elicit both systemic and mucosal responses, and TLR4 ligands usually represent powerful Th1 adjuvants. For example, nasal or oral administration of antigens with Monophosphoryl lipid A (MPL) or RC-529 (a synthetic MPL-like acylated monosaccharide) induces seric and local IgAs, as well as IFNγ-producing cytotoxic T lymphocytes (CTL) in mice [3,42,43]. The capacity of MPL to induce both IFNγ and IL10 production by immune cells is well documented [42,44]. MPL has been successfully used in humans in grass pollen specific subcutaneous immunotherapy and is currently tested in SLIT [45,46]. The co-administration of grass pollen allergens in combination with MPL enhances allergen-specific IgG1 and IgG4 antibody responses, with potentially induction of blocking antibodies which prevent histamine release by basophils and reduce IgE-mediated allergen presentation [47].

A triacylated pseudo-dipeptidic molecule, OM-197-MP-AC was also shown to increase antigen-specific Th1 responses in mice [35]. In addition, OM-197-MP-AC induces the maturation of human DCs, as well as their capacity to support the expansion of IFN-γ secreting T cells from naïve donors [34]. In this study, we analyzed OM-197-MP-AC as well as another triacylated pseudo-dipeptidic molecule, OM-294-BA-MP, as potential candidate adjuvants for allergy vaccines. Both OM-197-MP-AC and OM-294-BA-MP similarly induce murine and h-DCs maturation. However, the patterns of cytokine released are clearly distinct, since only OM-294-BA-MP stimulates IL1β, IL12p70, TNFα and IL10 production by DCs. Although, OM-197-MP-AC and OM-294-BA-MP-treated DCs do not alter transcription factors (i.e. Tbet, GATA3, Foxp3) specific for Th1, Th2 or regulatory T lymphocytes, respectively (Fig. 3), we do not exclude a potential transient up-regulation of those genes. However, OM-197-MP-AC treated-DCs elicits a moderate expression of the IFNγ gene by naïve T cells. As a Th1 adjuvant, it is nonetheless ineffective in reducing established AHR in mice sensitized with OVA. In contrast, OM-294-BA-MP treated-DCs trigger primary Th1/Treg responses with high IFNγ gene expression and a readily detectable induction of the IL10 gene. When administered sublingually in mice, OM-294-BA-MP reduces both AHR and lung inflammation.

Given the known effect of IFNγ in inducing tolerance in murine models of transplantation and autoimmune diseases [48—50], the relative efficacy of the two lipid A derivatives in our murine SLIT model might be related to their capacity to induce sufficient amounts of IFNγ by naïve T cells. IFNγ mediates tolerance by inhibiting Th2 effector T cells or inducing both nitric oxide synthetase (iNOS) and/or indoleamine-2,3-dioxygenase (IDO) expression in antigen presenting cell (APCs) [48—50]. As a non-mutually exclusive mechanism, the induction of the IL10 gene by OM-294-BA-MP is likely to be critical in the immunomodulatory function of this adjuvant. In support of this hypothesis, a diphosphorylated and triacetylated lipid A analogue, OM-174 which acts as a strong Th1 adjuvant in mice [51—53], had no impact on AHR in our SLIT model (data not shown). Also, we observed that only IL10-inducing adjuvants (e.g. selected strains of *Lactobacilli*, 1-α25-(dihydroxy)vitamin D3 plus dexamethasone [41] or the TLR2 ligand Pam3CSK4 (manuscript in preparation)) enhance SLIT efficacy in a murine model of established asthma.

The development of second-generation sublingual allergy vaccines, most particularly based on recombinant allergens, will likely rely upon safe and efficient adjuvants inducing T cell responses with a Th1/Treg profile. We conclude that selected synthetic triacyl lipid A derivatives such as OM-294-BA-MP represent valid adjuvants to improve SLIT in humans.

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