

Treatment with 8-OH-modified adenine (TLR7 ligand)-allergen conjugates decreases T helper type 2-oriented murine airway inflammation

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Summary

A strategy to improve allergen-specific immunotherapy is to employ new adjuvants stably linked to allergens. The study is addressed to evaluate the *in vivo* and *in vitro* effects of allergens [natural *Dermatophagoides pteronyssinus* 2 (*nDer p* 2) and ovalbumin (OVA)] chemically bound to an 8-OH-modified adenine. Humoral and cellular responses were analysed in allergen-sensitized and challenged mice by using conjugates (Conj) in a therapeutic setting. The *in vitro* activity of the conjugates on cytokine production induced by bone marrow dendritic cells and the co-culture system was also investigated. The *nDer p* 2-Conj treatment in *nDer p* 2-primed and challenged BALB/c mice reduced the numbers of eosinophils in bronchoalveolar lavage fluid and lung, airway allergen-driven interleukin-13 (IL-13) production in lung mononuclear cells and IgE, in comparison with *nDer p* 2-treated mice. The increase of IgG2a paralleled that of interferon- γ (IFN- γ) and IL-10 in allergen-stimulated spleen cells. Similar effects were elicited by treatment with OVA-Conj in an OVA-driven BALB/c model. The *nDer p* 2-Conj or OVA-Conj redirected memory T helper type 2 cells towards the production of IL-10 and IFN- γ also in C57BL/6 mice and when subcutaneously administered. Interleukin-10, IL-12 and IL-27 were produced *in vitro* by Conj-stimulated bone marrow dendritic cells, whereas IL-10 and IFN- γ were up-regulated in co-cultures of CD11c⁺ and CD4⁺ T cells from Conj-treated mice stimulated with allergen. Cytofluorometric analysis indicated that the Conj expanded IFN- γ - and IL-10- producing memory T cells. The Conj effects on IL-10^{-/-} and IL-12^{-/-} mice confirmed the role of IL-10 and IFN- γ in inducing a protective and balanced redirection the T helper type 2-mediated airway inflammation.

Keywords: adenine derivatives; adjuvants; immunotherapy; toll-like receptors; vaccines

Introduction

Although steroids, antihistamines and immunosuppressors usually control allergic diseases, specific immunotherapy is the only treatment able to modify the pathogenic mechanisms of these disorders. However, as

this treatment is inefficient in a high percentage of patients,¹ several approaches are under investigation to increase the efficiency of this procedure, including optimization of antigens, new administration routes and novel adjuvants.²⁻⁴ Among new adjuvants the use of low-molecular-weight synthetic compounds triggering

Abbreviations: BALF, bronchoalveolar lavage fluid; BMDC, bone marrow dendritic cells; Conj, conjugate; DC, dendritic cell; IFN, interferon; IL, interleukin; MNC, mononuclear cells; *nDer p* 2, natural *Dermatophagoides pteronyssinus* group 2 major allergen; OVA, ovalbumin; PAS, periodic acid Schiff; Th, T helper; TLR, toll-like receptor

endosomal Toll-like receptor (TLR) on dendritic cells (DC) has been proposed.^{5,6} Notably, the soluble ligands of endosomal TLR, in particular of TLR7, have been described as inducing *in vivo* autoantibodies and playing a still unclear role in autoimmunity.^{7,8} The chemical binding of allergens with adjuvant improves the activity of the constructs because it allows the delivery of the two components inside the same antigen-presenting cell with the outgrowth of the innate response redirecting the T helper type 2 (Th2) response. Hence the conjugate (Conj) with endosomal TLR ligands should increase safety by avoiding chronic B-cell activation and the onset of autoimmunity.⁹ The vaccination with the conjugate between ovalbumin (OVA) and 3M042 (an imidazoquinoline compound triggering TLR7/8) exerted a shift to a Th1 response, promoting antigen uptake by resident and migratory DC subsets.¹⁰ In atopic patients the major allergen in ragweed pollen (*Amb a 1*) stably bound to a 22-mer oligodesossinucleotide sequence (a TLR9 ligand) associated with improvement of symptoms, decreased IgE, produced a transient increase of specific IgG and, locally, eosinophils and Th2 cells.¹¹

We have previously shown that the mixture of an antigen with 2-,9-substituted 8-OH adenines influences the profile of T effector cells both in humans and mice.^{12,13} The Conj between SA26E, an 8-OH modified adenine triggering TLR7 in human and murine cells, and the natural group 2 major allergen (*nDer p 2*) from *Dermatophagoides pteronyssinus* was able to redirect allergen-specific Th2 responses *in vitro* and prevent the development of airway inflammation *in vivo*.¹⁴

The aim of this study was to evaluate the activity on murine airway inflammation of two (*nDer p 2* and OVA) conjugates in a therapeutic setting and to establish their mechanism of action. Even though some different effects were related to the structure of the two allergens, to mouse strain and to administration route, the results show that these novel conjugates deeply impair airway inflammation when administered in allergen-sensitized and challenged mice. The *in vitro* and *in vivo* data indicate that the major activity of these new compounds is to up-regulate and expand interferon- γ (IFN- γ) and interleukin-10 (IL-10) -producing memory T cells, a protective and balanced cytokine profile redirecting the allergen-specific Th2 response.

Materials and methods

Reagents

The LoToxTM *nDer p 2* used throughout the study was purchased from Indoor Biotechnologies Ltd. (batch no. 31059) (Charlottesville, VA) and certified to contain < 0.03 EU/ μ g endotoxin. Low-endotoxin RPMI-1640 medium (VLE-RPMI 1640; Biochrom AG, Berlin, Germany) was supplemented with low endotoxin 2 mM

L-glutamine, 2 mM 2-mercaptoethanol, 100 U/ml penicillin and 100 μ g/ml streptomycin (all from Sigma Chemical Co, Milan, Italy) (complete medium). Fetal calf serum was from HyClone (Thermo Scientific, Milan, Italy). Both PMA and ionomycin were purchased from Sigma-Aldrich (Milan, Italy). OVA and R-848 (Resiquimod, S28463) were from Invivogen (Milan, Italy). The endotoxin content of all the final reagents (allergens, SA26E and their conjugates) before use was assessed by LAL Test (Bio-Whittaker, Walkersville, MD), which was consistently lower than the detection limit of the assay. Anti-murine CD3 [phycoerythrin-allophycocyanin-conjugated (PE-APC)], CD4 (APC-FITC) and CD8 (PE) monoclonal antibodies were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Anti-murine IFN- γ (FITC) and anti-murine IL-13 (PE) were purchased from eBioscience (San Diego, CA). Anti-murine IL-10 (PE) was purchased from Becton-Dickinson (Mountain View, CA).

Synthesis of SA26E, preparation of OVA- and *nDer p 2*-Conj and stimulation of TLR-transfected HEK293

SA26E synthesis and its binding to OVA (or *nDer p 2*) were performed as previously described.¹⁴ Briefly, SA26E was dissolved in 130 μ l DMSO (Sigma-Aldrich) and chemically conjugated with 3 mg of purified OVA or 1.5 mg of *nDer p 2* in phosphate buffer (to final volumes of 1.5 and 3 ml, respectively) by overnight incubation at 4° with continuous rotation. Unconjugated SA26E was then removed by repeated dialysis (2000 molecular weight cut-off, Slide-A-Lyzer cassettes; Pierce, Rockford, IL) with PBS. Conjugates were divided into aliquots and stored at -20° until use.

HEK293 were transiently transfected with murine TLR7 and ELAM-1 promoter nuclear factor- κ B luciferase or with ELAM-1 promoter nuclear factor- κ B luciferase alone reporter plasmid.¹⁴ After transfection, cells were plated in 48 flat-bottomed plates in D-MEM (Sigma-Aldrich) supplemented with 2 mM L-glutamine and 5% FCS for 18 hr and then stimulated with R848 (6 μ M), SA26E (2 μ g), allergen (10 μ g/ml) and allergen-Conj (10, 2 and 0.4 μ g/ml) for an additional 18 hr. At the end of the culture, luciferase activity was determined in cell lysates by a luciferase Assay System (Promega, Madison, WI) (see Supplementary material, Fig. S1).

Mice

Interleukin-10-deficient mice (B6.129P2-*Il10*^{tm1Cgn}/J; stock #002251) and IL-12-deficient mice (B6.129S1-*Il12a*^{tm1Jm}/J; stock # 002692) were obtained from the Jackson Laboratory (Bar Harbor, ME). Both strains are on the C57BL/6 background. Female 6- to 8-week-old BALB/c and C57BL/6 mice were purchased from Charles River (Calco, Italy) and the animal study protocol was approved by the Institutional National guidelines and local animal ethics regulations.

In vivo therapeutic models

Mice were intraperitoneally sensitized at days 0 and 7 with 100 µl PBS containing *nDer p 2* or OVA (10 µg) adsorbed onto 2.25 µg Alum (Imject; Pierce). Negative controls were sham sensitized with PBS in Alum. Mice were challenged intratracheally at day 14 by administration of *nDer p 2* (10 µg in 50 µl of PBS) or PBS (negative control). Treatment consisted of administration of *nDer p 2*-Conj (intraperitoneally) or OVA-Conj (intraperitoneally or subcutaneously) or the corresponding allergen (10 µg in 100 µl of PBS), at 21, 23, 26 and 28 days. After the last administration of the adduct, mice were intratracheally re-challenged with allergens at 49 and 53 days and killed 3 days later for analysis (Fig. 1).^{15–17}

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed as previously described.¹³ BAL cells were morphologically characterized by Diff-Quick.

Lung histology

Lung sections were stained with haematoxylin and eosin and periodic acid Schiff (PAS) to evaluate lung inflammation, eosinophilic infiltration and goblet cell hyperplasia using previously described scores.^{14,18}

Generation of bone marrow dendritic cells

Bone marrow dendritic cells (BMDC) were prepared according to well-defined protocols¹⁹ and cultured *in vitro* at day 8 for 6 hr (mRNA detection) and 72 hr (protein detection) with OVA or *nDer p 2* (10 µg/ml), OVA-Conj or *nDer p 2*-Conj (10 µg/ml), or medium alone. At the end of the cultures, the cells were collected for total RNA extraction and supernatants were assayed for their IL-10, IL-12, IL-27, IL-1β, IL-23, CXCL10, IL-25, IL-6 and IFN-α content.

CD11c⁺ and CD4⁺ isolation and co-cultures

Dendritic cells and CD4⁺ T cells were purified from spleens of *nDer p 2*- or Conj-treated C57BL/6 mice by positive selection with anti-CD11c monoclonal antibodies bound to MACS Microbeads and a CD4⁺ T-cell Isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. The enrichment of isolated cells examined by cytometry was consistently > 95%. In the co-culture system purified CD11c⁺ spleen cells were co-cultured with purified CD4⁺ T cells (CD11c⁺ : CD4⁺ cell ratio 1 : 4) in the presence of medium or allergen or allergen-Conj at the final concentration of 50 µg/ml for 12 hr (mRNA detection) and 72 hr (protein detection).

Interleukin-10, IFN-γ and IL-13 production by co-cultures and purified CD4⁺ T cells was evaluated in flow cytometry with anti-CD3 APC, anti-IL-10 PE, anti-IFN-γ FITC and IL-13 PE. Stained cells were analysed on a BD-FACSCanto flow cytometer using the DIVA™ software (Becton Dickinson), as described previously.¹²

ELISA

Lung, spleen and mediastinal lymph node mononuclear cells (MNC) were prepared and cultured with allergens as previously described.¹³ Three-day culture supernatants were used for evaluating IFN-γ, IL-10 and IL-13 measured by commercial ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Detection limits for IFN-γ, IL-10 and IL-13 were 2, 4 and 1.5 pg/ml, respectively. BMDC stimulated with conjugates were assayed by commercially available ELISA kits (R&D Systems). Detection limits of IL-12, IL-27, IL-1β, IL-23, CXCL10, IL-25, IL-6 and IFN-α kits were 7.5, 19, 7.5, 4.8, 4.17, 4.2, 1.8 and 12.5 pg/ml, respectively.

Total and OVA-specific IgE, allergen-specific IgG1 and IgG2a antibodies were measured by ELISA as described.¹⁸

Quantitative mRNA analysis

Total lung RNA from snap-frozen mouse lungs were extracted using TRIzol reagent (RNAwiz; Invitrogen,

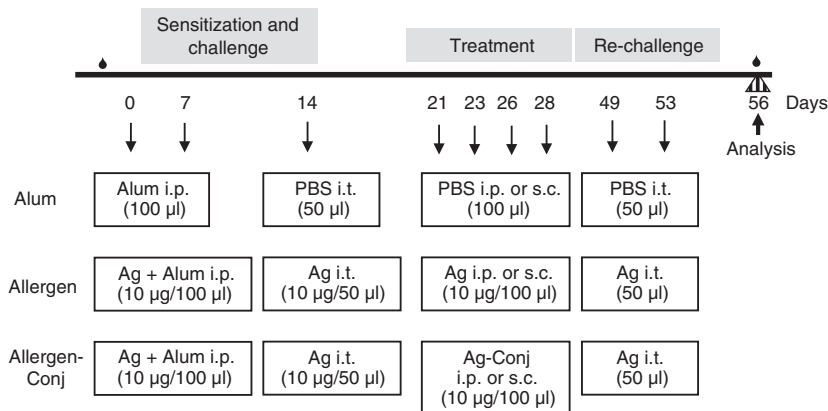


Figure 1. Experimental protocol. Sensitization, challenge, treatment and analysis timing in a murine model of airway inflammation. i.p., intraperitoneal; i.t., intratracheal; s.c., subcutaneous; Ag, allergen; ◆, blood sampling.

Milan, Italy), whereas total RNA from MNC were extracted using the RNeasy mini kit (Qiagen, Milan, Italy) as previously described.¹⁴ Real-time quantitative PCR was performed on an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Warrington, UK) with Applied Biosystems predesigned TaqMan Gene Expression assays and reagents according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using Student's *t*-test and analysis of variance (with Bonferroni correction). *P* values < 0.05 were considered significant.

Results

nDer p 2-Conj improves airway inflammation in a therapeutic setting

We assayed the effects of *nDer p 2*-Conj administration in allergen-immunized and challenged BALB/c mice, usually prone to induce a Th2-oriented response, using the therapeutic protocol described in the Fig. 1. Mice treated with *nDer p 2*-Conj showed a significant reduction of numbers of eosinophils and neutrophils in BAL fluid (BALF), with no changes of lymphocytes compared with *nDer p 2*-treated control mice (Fig. 2a). The lung histology indicated a reduced proportion of infiltrating eosinophils in both perivascular and peribronchial lung compartments of *nDer p 2*-Conj-treated mice (Fig. 2b). In the lung, the mRNA expression of the mucin-related gene *GOB-5*, as well as the PAS⁺ cellular scores, were reduced, but not significantly (see Supplementary material, Fig S2). Allergen-stimulated MNC from lung and mediastinal lymph nodes of *nDer p 2*-Conj-treated mice showed significant reduction of IL-13, with unchanged IFN- γ and IL-10 compared with MNC obtained from *nDer p 2*-treated mice (Fig. 2c left panel and data not shown). The reduction of IL-13 was however associated with the significant increase of mRNA expression of IFN- γ and IL-10 in spleen MNC (Fig. 2c right panel). Finally, in *nDer p 2*-Conj-treated mice, total IgE and *nDer p 2*-specific IgG1 were significantly reduced compared with *nDer p 2*-treated animals (Fig. 2d,e left panel), whereas allergen-specific IgG2a was significantly increased (Fig. 2e right panel).

OVA-Conj decreases airway inflammation in OVA-sensitized mice

We then asked whether another allergen (OVA), stably coupled to the same 8-OH-modified adenine, would display the *in vivo* effects described with *nDer p 2*-Conj in a therapeutic setting in BALB/c strain.

As established for *nDer p 2*-Conj,¹⁴ we initially showed that OVA-Conj was able to selectively stimulate TLR7-transfected HEK293 cells. Indeed the conjugates, but not the allergen alone, induced nuclear factor- κ B activity in transfected cells (see Supplementary material, Fig. S1).

We showed a significant reduction of the proportion but not the number of eosinophils in BALF of OVA-Conj-treated mice in comparison to OVA-treated animals ($40.35 \pm 0.51\%$ versus $29.85 \pm 3.92\%$ *P* < 0.02) (Fig. 3a). The conjugate had no effects on the proportion and numbers of other inflammatory cells in BALF (Fig. 3a). In addition, the treatment reduced the numbers of eosinophils in both peribronchial and perivascular lung compartments (Fig. 3b). In the lung, the *GOB-5* mRNA expression, as well as the PAS⁺ cellular scores, were reduced, even though not significantly (see Supplementary material, Fig. S2). Upon OVA stimulation, MNC from lung and mediastinal lymph nodes from OVA-Conj-treated mice showed significantly increased IFN- γ and decreased IL-13 compared with OVA-treated mice (Fig. 3c and data not shown). The OVA-Conj treatment decreased total and OVA-specific IgE and significantly increased allergen-specific IgG2a with no change of allergen-specific IgG1 (Fig. 3d,e).

The mouse strain and the route of administration do not influence the Conjugates' effects

To establish whether the redirection of the allergen-specific Th2 response to a more protective Th cell profile (IFN- γ and IL-10 production) was a general phenomenon, we performed the same experimental protocol with both compounds in C57BL/6 strain. *nDer p 2*- and OVA-Conj-treated C57BL/6 mice showed the same modifications as described for Conj-treated BALB/c mice (decreased numbers of eosinophils in BALF and lung, PAS infiltration scores, serum total IgE and allergen-specific IgG1 and increased allergen-specific IgG2a) (Fig. 4a and see Supplementary material, Fig. S2 and data not shown). Importantly, in these mice IFN- γ and IL-10 production by lung, mediastinal lymph nodes and spleen MNC upon the corresponding allergen stimulation were significantly increased and associated with a reduction (significant in *nDer p 2* but not in OVA model) of IL-13 (Fig. 4b).

To evaluate the effect of changing the route of administration, the subcutaneous injection of the conjugate was performed. In OVA-sensitized and challenged BALB/c or C57BL/6 mice, the subcutaneous treatment with OVA-Conj induced increased IFN- γ and IL-10 production by OVA-stimulated lung MNC compared with OVA-treated mice (Fig. 5a). These results paralleled the significant increase of specific IgG2a (Fig. 5b).

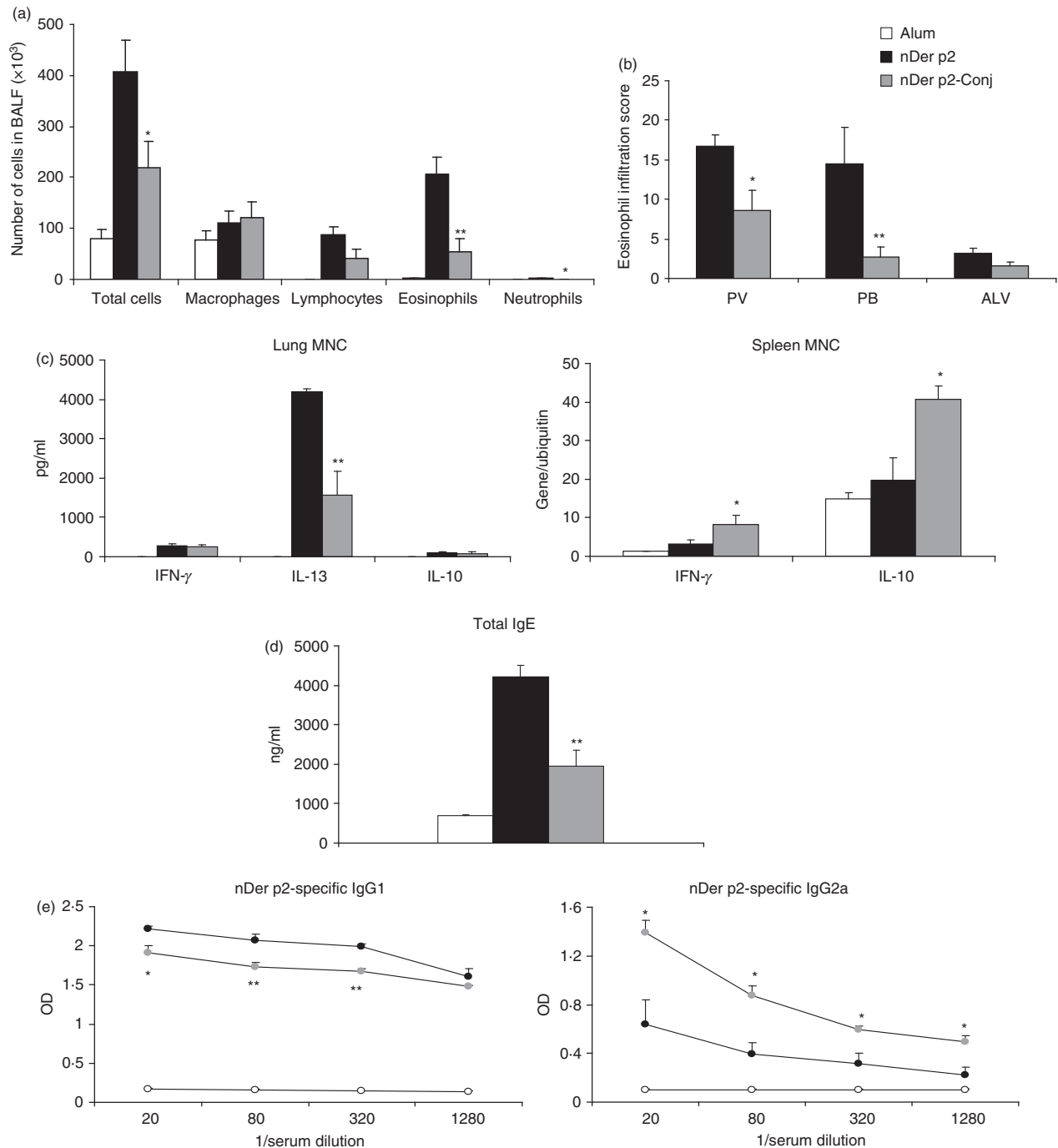


Figure 2. The *nDer p 2-Conj* down-regulates allergen-induced airway inflammation in BALB/c mice. (a) Differential cell counts in bronchoalveolar lavage fluid (BALF) performed 72 hr after the last challenge. (b) Eosinophil infiltration in lung compartments (PV, perivascular; PB, peribronchial; ALV, alveolar). (c) Cytokine production by lung mononuclear cells (MNC) upon *in vitro* *nDer p 2* stimulation (left panel) and cytokine mRNA expression in spleen MNC (right panel). (d, e) Detection of total IgE and allergen-specific IgG1 and IgG2a. All reported data are referred to pooled results from three separate experiments; six mice/group. Data are expressed as the mean (\pm SEM) and statistical significance between the allergen and allergen-Conj groups is reported. * $P < 0.05$, ** $P < 0.01$.

The conjugates induce pro-inflammatory and regulatory cytokines *in vitro*

To study the mechanisms down-regulating airway inflammation, we evaluated the activity of conjugates on

antigen-presenting cells *in vitro*. BMDC generated from wild-type BALB/c mice were cultured *in vitro* with *nDer p 2*- and OVA-Conj or the corresponding allergens. The conjugates induced higher levels of IL-10, IL-12, IL-27 (at the

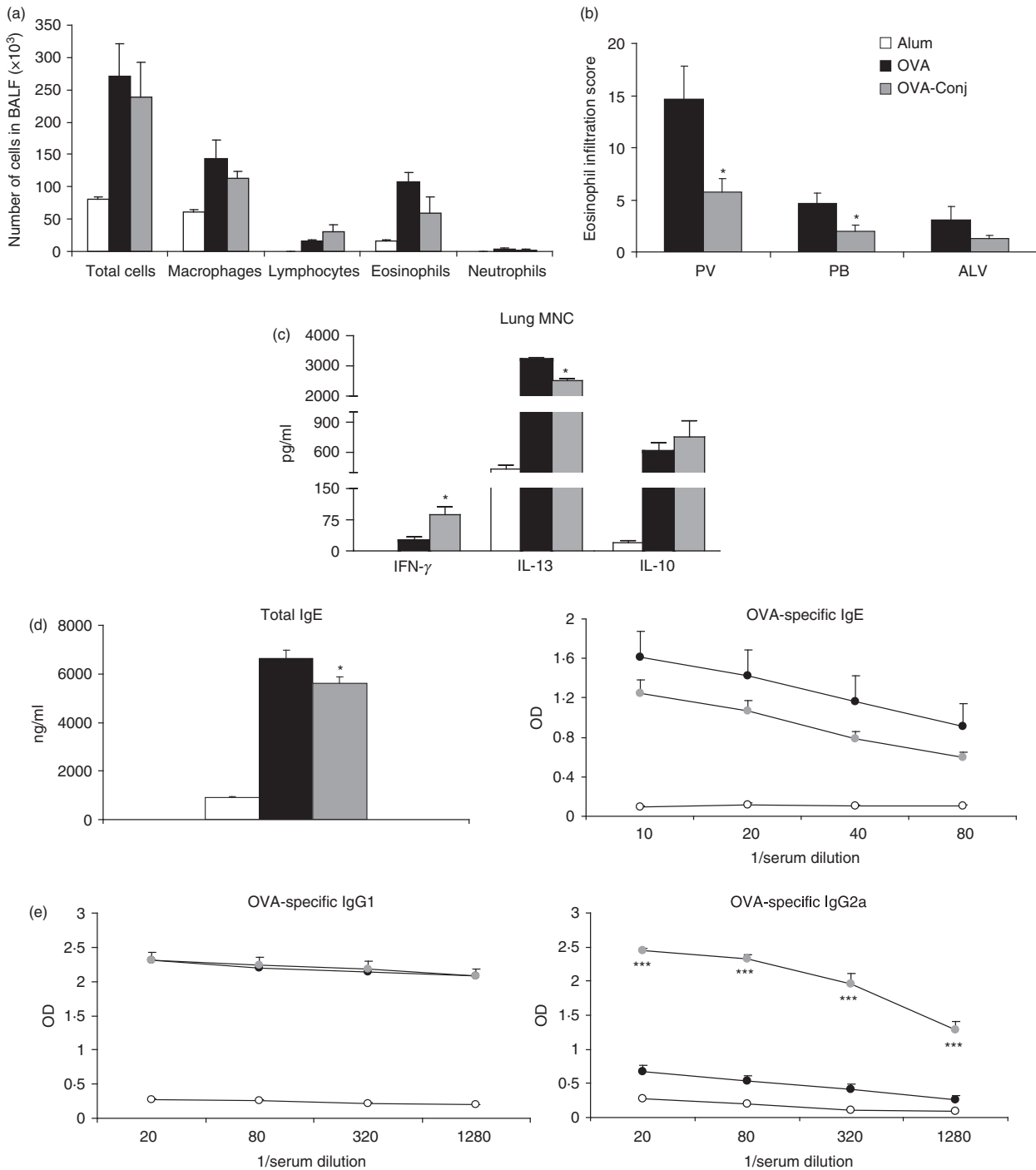


Figure 3. Effects of ovalbumin (OVA)-Conj treatment in BALB/c mice. (a) Differential cell counts in bronchoalveolar lavage fluid (BALF) performed 72 hr after the last challenge. (b) Eosinophil infiltration in lung compartments (PV, perivascular; PB, peribronchial; ALV, alveolar) (c) Cytokine production upon *in vitro* OVA stimulation of lung mononuclear cells (MNC). (d, e) Detection of total IgE and allergen-specific IgE, IgG1 and IgG2a. All reported data refer to pooled results from three separate experiments; six mice/group. Data are expressed as the mean (\pm SEM) and statistical significance between the allergen and allergen-Conj groups is reported. * $P < 0.05$, *** $P < 0.001$.

mRNA and protein levels) than those elicited by allergens alone (Table 1). BMDC generated from wild-type C57BL/6 mice gave similar results (data not shown). Other secreted

molecules such as IL-1 β , IL-23, CXCL10 were up-regulated in BMDC stimulated by both conjugates whereas IL-6, IL-25 and type I IFN were unaffected (data not shown).

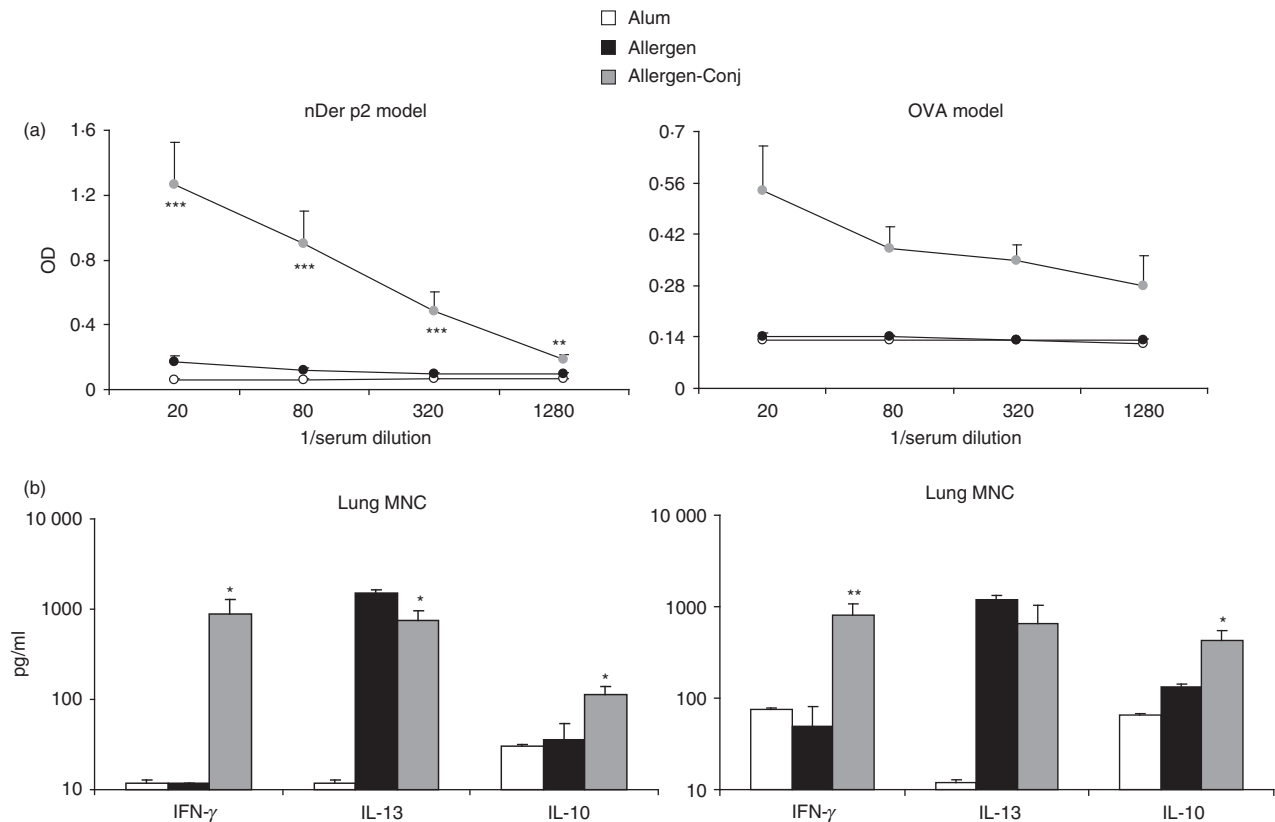


Figure 4. Effects of conjugates treatment in C57BL/6 mice. (a) Detection of allergen-specific IgG2a in *nDer p 2-Conj-* (left panel) and ovalbumin (OVA)-Conj- (right panel) treated mice. (b) Cytokine production by lung mononuclear cells (MNC) upon *in vitro* allergen stimulation in *nDer p 2-Conj-* treated mice (left panel) and OVA-Conj-treated mice (right panel). All reported data refer to pooled results from four separate experiments; six mice/group. Data are expressed as the mean (\pm SEM) and the statistical significance between the allergen and allergen-Conj groups is reported. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

The *in vivo* effects of conjugates require the production of both IFN- γ and IL-10

To better define the role of inflammatory and regulatory cytokines, we compared the *in vivo* effects of *nDer p 2-Conj* treatment in IL-10^{-/-} and IL-12^{-/-} mice.

In IL-10^{-/-} mice, specific IgG2a was significantly increased and total IgE was inhibited in *nDer p 2-Conj*-treated mice compared with *nDer p 2*-treated mice, whereas the IgG1 was unchanged. In IL-12^{-/-} mice, *nDer p 2-Conj* treatment significantly increased IgE and allergen-specific IgG1 compared with allergen-treated mice (Fig. 6a).

The proportions and numbers of lymphocytes were significantly increased in BALF from *nDer p 2-Conj*-treated IL-10^{-/-} ($29\,290 \pm 6425$ versus 2602 ± 1103 , $P < 0.01$) and IL-12^{-/-} ($12\,474 \pm 5310$ versus 182 ± 119 , $P < 0.05$) in comparison with *nDer p 2*-treated IL-10^{-/-} and IL-12^{-/-} mice, whereas both eosinophils and neutrophils were unchanged (data not shown). Moreover, lung histological peribronchial and perivascular infiltration and eosinophil scores were significantly increased in IL-10^{-/-} and IL-12^{-/-} Conj-treated mice compared with

allergen-treated mice (Fig. 6b). In addition, PAS score was significantly higher in Conj-treated than in *nDer p 2*-treated IL-12^{-/-} mice (1.518 ± 0.215 versus 0.308 ± 0.117 , $P < 0.01$). On the other hand, PAS score was unchanged in Conj-treated IL-10^{-/-} and wt mice compared with allergen-treated animals (data not shown). The most important finding is that IFN- γ and IL-13 were strongly up-regulated in the allergen-stimulated MNC from lung, mediastinal lymph nodes and spleen of *nDer p 2-Conj*-treated compared with *nDer p 2*-treated IL-10^{-/-} mice (Fig. 6b). Of note, these cytokines were significantly higher than those detected in Conj-treated IL-12^{-/-} mice (Fig. 6c).

Lastly to establish whether the conjugates expand allergen-specific T cells producing IL-10 and IFN- γ , we performed co-cultures between purified allergen-pulsed splenic CD11c⁺ and CD4⁺ T cells derived from treated C57BL/6 mice. As expected, at the mRNA and protein levels, IL-10 and IFN- γ were higher in co-culture supernatants from *nDer p 2-Conj-* than *nDer p 2*-treated mice (Fig. 7a). Accordingly, the cytometric analysis on the 3-day-cultured CD3-gated cells of the previous

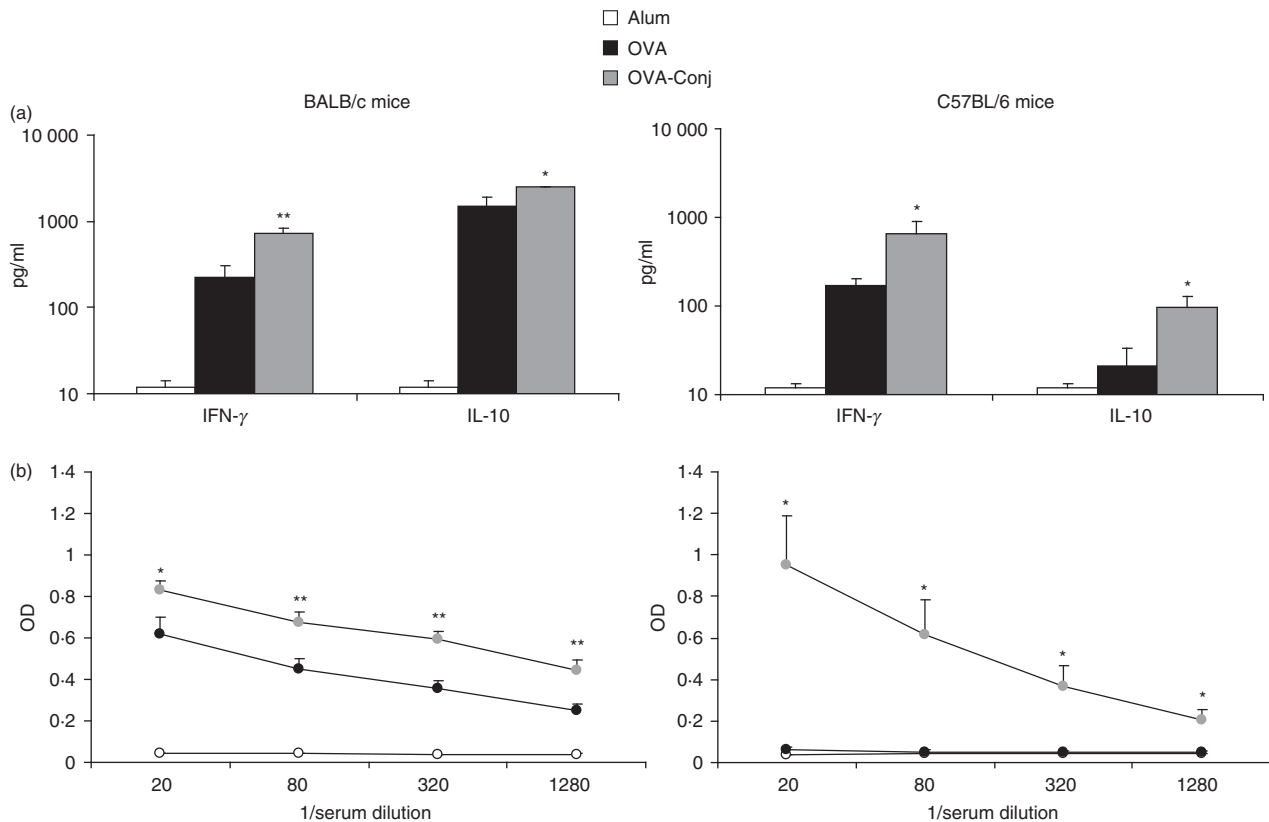


Figure 5. Effects of ovalbumin (OVA)-Conj administered subcutaneously. (a) Cytokine production of lung mononuclear cells (MNC) from Conj-treated-BALB/c (left panel) and Conj-treated-C57BL/6 (right panel) mice upon *in vitro* OVA stimulation. (b) Effects of subcutaneous treatment with OVA-Conj on serum levels of OVA-specific IgG2a in both mouse strains. All reported data refer to pooled results from three separate experiments; six mice/group. Data are expressed as the mean (\pm SEM) and statistical significance between the allergen and allergen-Conj groups is reported. * $P < 0.05$, ** $P < 0.01$.

experiment indicates a significantly increased proportion of IL-10- and IFN- γ -producing cells in cultures from Conj-treated mice in comparison with allergen-treated mice (Fig. 7b). Moreover, upon 6-hr PMA/ionomycin stimulation the *ex vivo* purified spleen CD4⁺ T cells from *nDer p 2*-Conj-treated mice also expressed higher proportions of IL-10- and IFN- γ -producing cells than those from control mice (Fig. 7b). In both experimental conditions, IL-13-producing T cells were significantly reduced in Conj-treated mice (Fig. 7b).

Discussion

Specific immunotherapy represents a relevant approach to cure respiratory allergy because it is the only treatment shown to modulate pathogenetic Th2 responses to allergens.^{20–22} We recently showed that the chemical conjugation between *nDer p 2* and an 8-OH modified adenine triggering TLR7, called SA26E, is able to improve the vaccine construct and to reduce systemic exposure, targeting appropriate antigen-presenting cells, and so resulting in a more effective modulation of allergen-specific T-cell responses.¹⁴

In this paper, we provide evidence that the 8-OH-adenine conjugates administered in a therapeutic setting, mimic the therapeutic effects of specific immunotherapy in allergic patients by improving airway inflammation in allergen-sensitized mice, mainly through the up-regulation of IFN- γ and IL-10 by memory T cells.

The effects of previously described *nDer p 2*-Conj in *nDer p 2*-sensitized and challenged BALB/c mice, a Th2-prone strain, induced the impairment of inflammation as shown by the reduced eosinophils in BALF and lung and of mucin-secreting cells and their related genes in lung. Moreover, the significant decrease of type 2 cytokines (namely IL-13) produced by allergen-stimulated MNC from lung, mediastinal lymph nodes and spleen in treated mice reflected the parallel reduction of *nDer p 2*-specific IgG1 and total IgE. These data indicate that the *nDer p 2*-Conj prevalently inhibits allergen-specific Th2 cells in the lungs of BALB/c mice. These data partially agree with those of Coffman and co-workers,²³ who described the selective inhibition of a ragweed-specific Th2 response and IgE in ragweed-primed BALB/c mice when they were treated intratracheally with a synthetic TLR9 ligand, without any increase of IFN- γ - or

Table 1. Cytokine mRNA expression and production by bone marrow dendritic cells from BALB/c mice

	Interleukin-10 (Gene/Ubiquitin)	Interleukin-12	Interleukin-27
OVA	0.4 ± 0.05	2.5 ± 0.4	1.2 ± 0.08
OVA-Conj	14.3 ± 3*	10.5 ± 1*	23 ± 4*
<i>nDer p 2</i>	0.5 ± 0.1	0.3 ± 0.01	0.3 ± 0.05
<i>nDer p 2</i> -Conj	4.2 ± 0.6*	4.3 ± 1.2	5.9 ± 1.8

	Interleukin-10 (pg/ml)	Interleukin-12	Interleukin-27
OVA	7.5 ± 7	4.5 ± 2.5	5.5 ± 5
OVA-Conj	375 ± 75*	151 ± 50	249 ± 50*
<i>nDer p 2</i>	9 ± 8	7.5 ± 7	8 ± 6
<i>nDer p 2</i> -Conj	120 ± 30	77 ± 22	150 ± 52

Cytokine mRNA expression was evaluated by real-time quantitative PCR. Cytokine production was measured using ELISA commercial kits. Data are expressed as mean values (\pm SEM) of gene/ubiquitin ratios or of proteins (above the levels of cultures with medium alone) in culture supernatants. All reported data are from pooled results of two separate experiments. Statistical significance between the allergen and the conjugate group is reported.

* $P < 0.05$.

IL-10-producing cells. In the present paper we confirm the down-regulation of IL-13 and total IgE upon conjugate treatment and we also show that *nDer p 2*-Conj shifts the allergen-specific Th2 cells towards an IFN- γ - and IL-10-oriented profile in allergen-stimulated spleen cells with a parallel significant increase of allergen-specific IgG2a in the serum. Finally, the *nDer p 2*-Conj induced *in vitro* pro-inflammatory (IL-12 and IL-27) and regulatory (IL-10) cytokines by BMDC derived from BALB/c mice. Taking into account that IFN- γ favours the isotype switch to IgG2a and directly inhibits IgE-producing B cells, these data suggest that the improvement of airway inflammation in BALB/c mouse models reflects the redirection of Th2 cells to a protective IFN- γ - and IL-10-oriented response rather than the selective inhibition of Th2 cells. Of note, in another strain (C57BL/6) more prone to favour the Th1 response, we confirmed that *nDer p 2*-Conj was mainly associated with the shift of the Th2 cells towards a pronounced IFN- γ and IL-10 cell profile in the lung.

This concept was confirmed by the data obtained with another allergen (OVA) bound to the same adjuvant (SA26E), which triggers lymphoid cells through TLR7. When OVA-sensitized and challenged BALB/c mice were treated with OVA-Conj, the decrease of eosinophils in BALF associated with reduced lung infiltration and eosinophil scores. Importantly, the reduction of total and OVA-specific IgE in OVA-Conj-treated mice was associated with the redirection of Th2 to IFN- γ -producing cells in the lung MNC stimulated with OVA, without any

significant increase of IL-10. Again, the imbalance of type 2 and type 1 cytokines was more evident in C57BL/6 mice treated by the same protocol in which, however, the increase of IL-10 was clearly significant in allergen-stimulated lung MNC. Besides the differences of the mouse strain, the type of allergens may also influence the *in vivo* effects of conjugates. In fact in the same strain (BALB/c) OVA-Conj-treated mice consistently showed higher levels of allergen-specific IgG2a than *nDer p 2*-Conj-treated mice.

All these findings not only suggest the importance of the mouse strain used but also reflect the differences between the two allergens in eliciting the T-cell response. *nDer p 2*, a selective inducer of the Th2 response, is chronically inhaled because the dust mite usually contaminates the environment. Hence, *nDer p 2* cannot be considered a neo-antigen, because the *in vivo* models can be influenced by the unpredictable inhaled dose of allergen. By contrast, OVA is more prone to expand IL-4- and IFN- γ -producing T cells and, if administered with adjuvant, it must be considered a true neo-antigen.^{13,24}

Another important topic of the paper is that the subcutaneous administration route, which is traditionally used in human specific immunotherapy, does not deeply influence the effects of conjugates. The subcutaneous protocol with OVA-Conj increased in lung MNC from BALB/c and C57BL/6 mice both for IFN- γ and IL-10 (not significantly in C57BL/6) and for allergen-specific IgG2a. Notably, even though the subcutaneous administration of antigen has been described to improve the Th17 response in some *in vivo* models,²⁵ IL-17A or related cytokines were never detected in the OVA-stimulated lung MNC by using the present protocols. This finding agrees with our previous results that the 8-OH-modified adenine-driven IL-10 and IL-27 are the best inhibitors of the Th17 response.²⁶

Overall, these data clearly show that these novel conjugates improve airway allergic inflammation irrespective of experimental models by modifying lung and serum parameters, whose degree is partially influenced by mouse strain, type of allergens and administration route, as previously reported.^{13,27-29}

More importantly, regardless of the previous different conditions employed, the paper provides a clear-cut demonstration that the efficacy of the new compounds resides in their ability to up-regulate IFN- γ and IL-10 *in vitro* and *in vivo*.

By using purified BMDC from wild-type BALB/c and C57BL/6 mice, we showed that IL-10 and type 1-inducing (IL-12, IL-27) molecules were highly stimulated by both conjugates compared with allergens alone. Although the conjugates *in vitro* also stimulate IL-1 β and IL-23 from BMDC, two cytokines that are essential to the development of Th17 responses, they consistently failed to promote the *in vivo* expansion of harmful Th17 cells because,

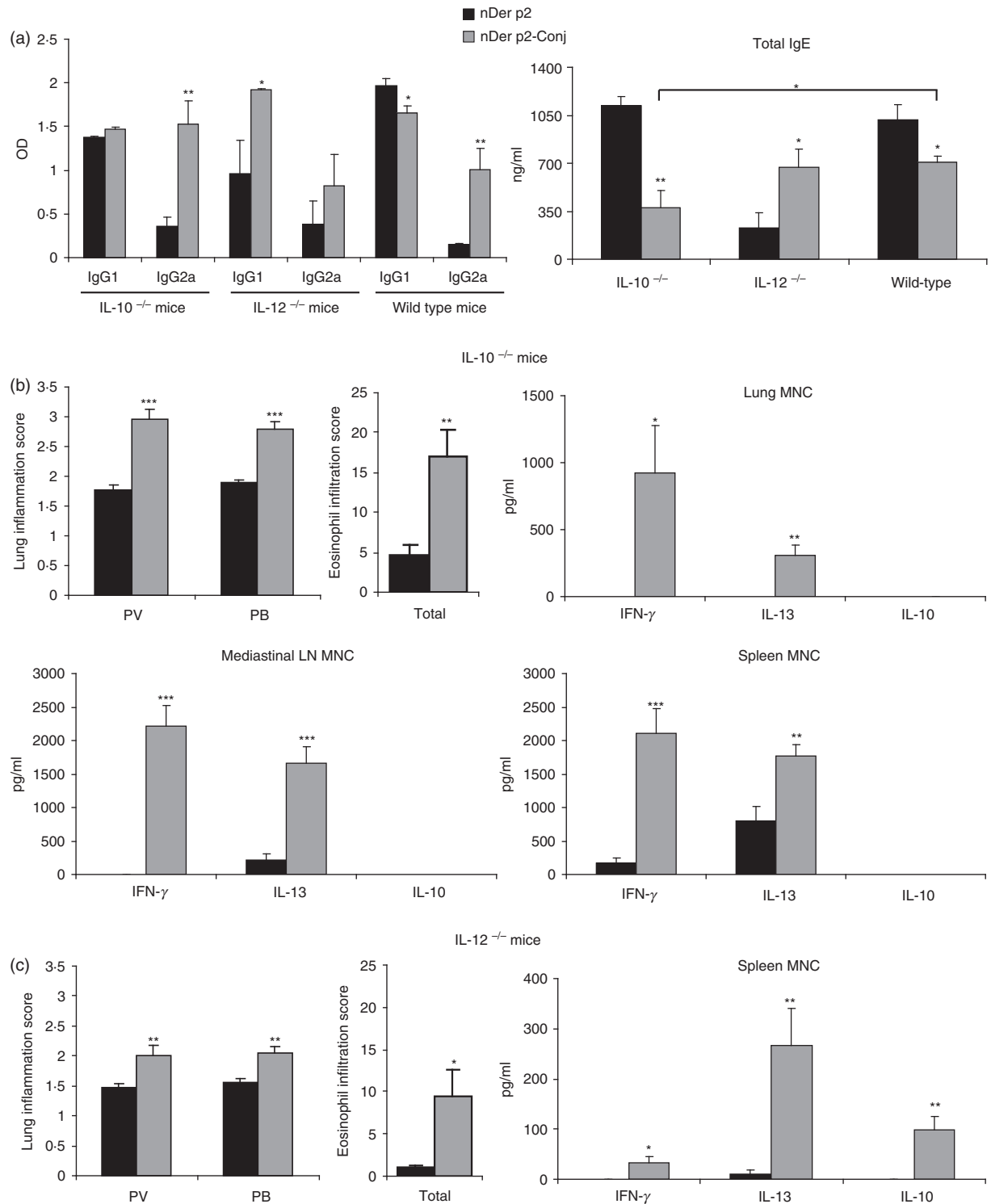
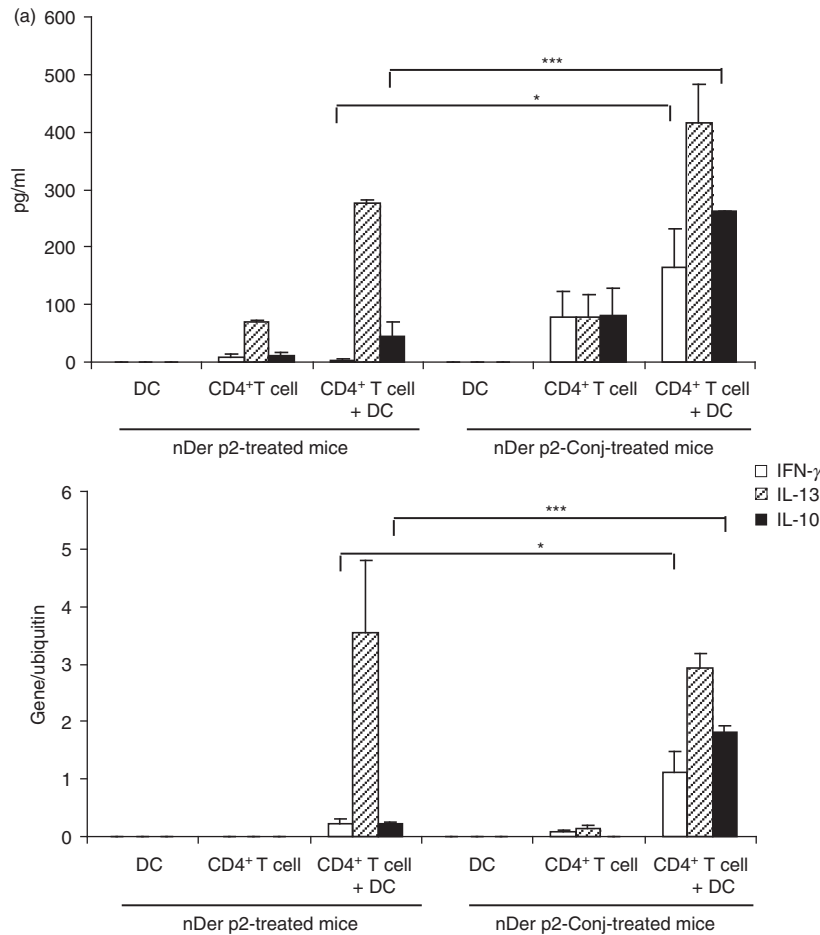


Figure 6. Modulatory effects of *nDer p 2-Conj* on airway inflammation in IL10^{-/-} and IL-12^{-/-} mice. (a) Detection of allergen-specific IgG1 and IgG2a (left panel) and total IgE (right panel) in the serum of IL-10^{-/-}, IL-12^{-/-} and wild-type C57BL/6 mice. (b) Lung inflammation scores (PV, perivascular; PB, peribronchial) and total eosinophil infiltration as described in Materials and methods. Cytokine production by lung, mediastinal lymph nodes and spleen mononuclear cells (MNC) upon *in vitro* *nDer p 2* stimulation in IL-10^{-/-} mice. (c) Lung inflammation scores (PV, perivascular; PB, peribronchial) and total eosinophil infiltration as described in Materials and methods. Cytokine production by spleen MNC upon *in vitro* *nDer p 2* stimulation in IL-12^{-/-} mice. All reported data refer to pooled results from two separate experiments; six mice/group. Data are expressed as the mean (\pm SEM) and statistical significance between the allergen and allergen-Conj groups is reported. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.



(b) Cytometric analysis of cytokines production by T cells

	% of CD4 ⁺ producing cytokines <i>ex vivo</i>		
	IFN- γ	IL-13	IL-10
nDer p2	1.52 \pm 0.29	3.45 \pm 0.08	1.32 \pm 0.10
nDer p2-Conj	3 \pm 0.50*	1.75 \pm 0.66*	2.12 \pm 0.26**
	% of CD4 ⁺ producing the indicated cytokines in the co-culture system		
	IFN- γ	IL-13	IL-10
nDer p2	0.58 \pm 0.08	3.1 \pm 0.1	0.64 \pm 0.04
nDer p2-Conj	1.53 \pm 0.06***	1.5 \pm 0.1**	1.02 \pm 0.07***

Figure 7. The conjugate induces interleukin-10 (IL-10) and interferon- γ (IFN- γ) production by allergen-stimulated T cells. (a) Cytokine production upon *in vitro* nDer p 2 stimulation in co-culture between purified spleen CD11c⁺ and CD4⁺ T cells obtained from nDer p 2- or nDer p 2-Conj-treated C57BL/6 mice (upper panel). Cytokine mRNA expression in cells *in vitro* cultured upon the same experimental conditions (lower panel). (b) IL-10-, IFN- γ - and IL-13-producing cells in CD3-gated cells co-cultured as reported in (a) or in purified CD4⁺ T cells from Conj- or allergen-treated mice stimulated with PMA/ionomycin. All reported data are referred to pooled results from two separate experiments; six mice/group. Data are expressed as the mean (\pm SEM) and statistical significance between the allergen and allergen-Conj groups is reported. * P < 0.05, ** P < 0.01, *** P < 0.001.

as previously analysed, they prevalently stimulate IL-17-inhibiting cytokines as IL-10 and IL-27.^{26,30}

The *in vivo* results from IL-10^{-/-} and IL-12^{-/-} mice clearly indicate that conjugate usually induces a strong IL-10 production which is not always detectable in different models, that partially inhibits IFN- γ - and IL-13-producing memory T cells. Indeed very high levels of both IFN- γ and IL-13 are produced by allergen-stimulated MNC from lung, mediastinal lymph nodes and spleen in nDer p 2-Conj-treated IL-10^{-/-} mice, but not by the same mice treated with allergen alone. Hence in these

mice the up-regulation of IFN- γ and IL-13 seems to be a feature of the conjugate but not of unbound allergen. Moreover, in Conj-treated IL-10^{-/-} mice, the effects of nDer p 2-driven IFN- γ (as the increased allergen-specific IgG2a, the IgE reduction due to the negative activity of the cytokine on IgE-producing B cells and the increased airway infiltration by neutrophils and lymphocytes) partially overcome those of IL-13 (regulating the number of PAS⁺ cells and eosinophils in BALF and lung). On the other hand, in the presence of an IFN- γ defect, as in IL-12^{-/-} mice, the IL-10 induced by the conjugate did not

completely block the effects of IL-13 production, as the increased IgE, eosinophils and PAS⁺ cells in the lung.

Lastly, the cytofluorimetric analysis on *ex vivo* purified CD4⁺ T cells or *in vitro* CD3-gated cells from co-cultured DC and T cells from the treated mice, provides evidence that the conjugates expand *in vivo* memory T cells producing IL-10 and IFN- γ .

Overall these data suggest that conjugate-induced IFN- γ and IL-10 are both essential to ameliorate the airway inflammation. Indeed, the former may redirect the allergen-mediated type 2 cytokines and IL-10 may quench the inflammatory effects of IFN- γ , potentially being able to promote autoimmunity.

The effects of Conj-induced IL-10 on Th17 and Th1 responses may explain why conjugates fail to induce autoantibodies in short priming¹⁴ or long therapeutic settings and to improve spontaneous diabetes in NOD mice (E. Maggi, unpublished data). The shift of the Th2 response to a protective and balanced T-cell profile is a valid reason to include the 8-OH-modified adenines among the other TLR ligands to be bound to allergens in novel specific immunotherapies.⁶ The use of conjugates between proteins linked to these types of adjuvant also represents a good strategy to design novel vaccine formulations for diseases in which a powerful and balanced Th1/regulatory type 1 T cell response is required.

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Author's contribution

F.N., S.P. and G.P. contributed to the conception and design of the study, data generation, analysis and interpretation of the data, and preparation of the manuscript. L.F., E.C. contributed to the design of the study, data generation, and the analysis and interpretation of the data. A.C., E.G.O., L.C. and D.B. contributed to data generation. S.R., P.P. and E.M. contributed to the conception and design of the study, data generation, and critical revision of the manuscript. A.V. contributed to the conception and design of the study, data generation, analysis and interpretation of the data, and preparation and critical revision of the manuscript.

Disclosure

E.G.O., S.R., E.M. and P.P. have a patent with the University of Florence and Azienda Ospedaliera -Universitaria Careggi. The rest of the authors declare that they have no relevant conflicts of interest.

References

- Maggi E, Vultaggio A, Matucci A. T-cell responses during allergen-specific immunotherapy. *Curr Opin Allergy Clin Immunol* 2012; **12**:1–6.
- Senti G, Prinz Vavricka BM, Erdmann I *et al*. Intralymphatic allergen administration renders specific immunotherapy faster and safer: a randomized controlled trial. *Proc Natl Acad Sci USA* 2008; **105**:17908–12.
- Broide DH. Immunomodulation of allergic disease. *Annu Rev Med* 2009; **60**:279–91.
- Linhart B, Valenta R. Mechanisms underlying allergy vaccination with recombinant hypoallergenic allergen derivatives. *Vaccine* 2012; **30**:4328–35.
- Akira S. Innate immunity and adjuvants. *Philos Trans R Soc Lond B Biol Sci* 2011; **366**:2748–55.
- Adner M, Starkhammar M, Georén SK, Dahlén SE, Cardell LO. Toll-like receptor (TLR) 7 decreases and TLR9 increases the airway responses in mice with established allergic inflammation. *Eur J Pharmacol* 2013; **718**:544–51.
- Matesic D, Lenert A, Lenert P. Modulating toll-like receptor 7 and 9 responses as therapy for allergy and autoimmunity. *Curr Allergy Asthma Rep* 2012; **12**:8–17.
- Krieg AM, Vollmer J. Toll-like receptors 7, 8, and 9: linking innate immunity to autoimmunity. *Immunol Rev* 2007; **220**:251–69.
- Rappuoli R, Mandl CW, Black S, De Gregorio E. Vaccines for the twenty-first century society. *Nat Rev Immunol* 2011; **11**:865–72.
- Kastenmüller K, Wille-Reece U, Lindsay RW *et al*. Protective T cell immunity in mice following protein-TLR7/8 agonist-conjugate immunization requires aggregation, type I IFN, and multiple DC subsets. *J Clin Invest* 2011; **121**:1782–96.
- Tulic MK, Fiset PO, Christodoulopoulos P *et al*. Amb a 1-immunostimulatory oligodeoxynucleotide conjugate immunotherapy decreases the nasal inflammatory response. *J Allergy Clin Immunol* 2004; **113**:235–41.
- Fili L, Ferri S, Guarna F *et al*. Redirection of allergen-specific TH2 responses by a modified adenine through Toll-like receptor 7 interaction and IL-12/IFN release. *J Allergy Clin Immunol* 2006; **118**:511–7.
- Vultaggio A, Nencini F, Fitch PM *et al*. Modified adenine (9-benzyl-2-butoxy-8-hydroxyadenine) redirects Th2-mediated murine lung inflammation by triggering TLR7. *J Immunol* 2009; **182**:880–9.
- Fili L, Vultaggio A, Cardilicchia E *et al*. A novel allergen-adjuvant conjugate suitable for specific immunotherapy of respiratory allergy. *J Allergy Clin Immunol* 2013; **S0091-6749**:00190–5.
- Santeliz JV, Van Nest G, Traquina P, Larsen E, Wills-Karp M. Amb a 1-linked CpG oligodeoxynucleotides reverse established airway hyperresponsiveness in a murine model of asthma. *J Allergy Clin Immunol* 2002; **109**:455–62.
- Kitagaki K, Businga TR, Kline JN. Oral administration of CpG-ODNs suppresses antigen-induced asthma in mice. *Clin Exp Immunol* 2006; **143**:249–59.
- Sel S, Wegmann M, Sel S, Bauer S, Garn H, Alber G, Renz H. Immunomodulatory effects of viral TLR ligands on experimental asthma depend on the additive effects of IL-12 and IL-10. *J Immunol* 2007; **178**:7805–13.
- Leech MD, Benson RA, De Vries A, Fitch PM, Howie SE. Resolution of Der p 1-induced allergic airway inflammation is dependent on CD4⁺CD25⁺Foxp3⁺ regulatory cells. *J Immunol* 2007; **179**:7050–8.
- Boonstra A, Asselin-Paturel C, Gilliet M, Crain C, Trinchieri G, Liu YJ, O'Garra A. Flexibility of mouse classical and plasmacytoid-derived dendritic cells in directing T helper type 1 and 2 cell development: dependency on antigen dose and differential toll-like receptor ligation. *J Exp Med* 2003; **197**:101–9.
- Romagnani S. T-cell responses in allergy and asthma. *Curr Opin Allergy Clin Immunol* 2001; **1**:73–8.
- Larché M. Peptide-based immunotherapy: new developments. *Arb Paul Ehrlich Inst Bundesamt Sera Impfstoffe Frankf A M* 2003; **94**:133–9.
- Fiocchi A, Annunziato F, Assa'ad A *et al*. The management of paediatric allergy: not everybody's cup of tea – 10–11th February 2012. *Curr Opin Allergy Clin Immunol* 2013; **13**(Suppl 1):S1–50.
- Hessel EM, Chu M, Lizcano JO *et al*. Immunostimulatory oligonucleotides block allergic airway inflammation by inhibiting Th2 cell activation and IgE-mediated cytokine induction. *J Exp Med* 2005; **202**:1563–73.
- Du Q, Zhou LF, Chen Z, Gu XY, Huang M, Yin KS. Imiquimod, a toll-like receptor 7 ligand, inhibits airway remodelling in a murine model of chronic asthma. *Clin Exp Pharmacol Physiol* 2009; **36**:43–8.
- He R, Kim HY, Yoon J *et al*. Exaggerated IL-17 response to epicutaneous sensitization mediates airway inflammation in the absence of IL-4 and IL-13. *J Allergy Clin Immunol* 2009 Oct; **124**:761–70.
- Vultaggio A, Nencini F, Pratesi S *et al*. The TLR7 ligand 9-benzyl-2-butoxy-8-hydroxy adenine inhibits IL-17 response by eliciting IL-10 and IL-10-inducing cytokines. *J Immunol* 2011; **186**:4707–15.
- Lombardi V, Van Overtvelt L, Horiot S *et al*. Toll-like receptor 2 agonist Pam3CSK4 enhances the induction of antigen-specific tolerance via the sublingual route. *Clin Exp Allergy* 2008; **38**:1819–29.

- 28 Moingeon P, Lombardi V, Saint-Lu N, Tourdot S, Bodo V, Mascarell L. Adjuvants and vector systems for allergy vaccines. *Immunol Allergy Clin North Am* 2011; **31**: 407–19.
- 29 Creticos PS, Schroeder JT, Hamilton RG *et al.* Immunotherapy with a ragweed-toll-like receptor 9 agonist vaccine for allergic rhinitis. *N Engl J Med* 2006; **355**:1445–55.
- 30 Vultaggio A, Nencini F, Pratesi S, Petroni G, Romagnani S, Maggi E. Poly(I:C) promotes the production of IL-17A by murine CD1d-driven invariant NKT cells in airway inflammation. *Allergy* 2012; **67**:1223–32.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Conjugates trigger murine Toll-like receptor 7.

Figure S2. Effects of conjugates on goblet cell hyperplasia.