



Dermatophagoides pteronyssinus group 2 allergen bound to 8-OH modified adenine reduces the Th2-mediated airway inflammation without inducing a Th17 response and autoimmunity

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ABSTRACT

8-OH modified adenine bound to Dermatophagoides pteronyssinus group 2 (nDer p2-Conj), a novel allergen-TLR7 agonist conjugate, improves murine airway inflammation in priming and therapeutic settings, however no data are known on the activity of this construct on Th17 cells.

The aim of the study was to evaluate if nDer p2-Conj elicited *in vivo* Th17 cells and Th17-driven autoimmune responses, by using both short- and long-term priming and therapeutic protocols in a nDer p2-driven model of murine airway inflammation. The conjugate induced the *in vitro* production of cytokines favouring the Th17 polarization by bone marrow-derived dendritic cells. In short-term protocols, the priming or treatment with the conjugate ameliorated the airway inflammation by shifting Th2 allergen-specific cells into T cells producing IFN- γ , IL-10, but not IL-17A. Similar results were found in long-term protocol where the conjugate down-regulated airway inflammation without any evidence of autoimmune response and B cell compartment expansion. nDer p2-Conj also failed to shorten the spontaneous onset of diabetes on conjugates-primed NOD/LtJ mice. We found that neutrophils in BALF, ROR- γ t and IL-17A expression in lungs were increased in conjugate-treated IL-10KO mice. These data emphasize the role of conjugate-driven IL-10 production, which can regulate the activity of memory Th17 cells and prevent the onset of autoimmune response.

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1. Introduction

Respiratory allergy is a chronic disease mainly mediated by Th2 cells-driven pathogenetic mechanism (Fahy, 2015). Recent reports provide clear-cut evidence that a Th17 response is also involved in chronic asthma mainly in the severe, steroid-resistant phenotype with a prevalent neutrophilic lung infiltration (Cosmi et al., 2011; Barnes, 2015). Thus, allergy immunotherapy (AIT), which is the

only treatment able to modify the pathogenic mechanisms of these disorders, should redirect not only Th2 response but also inhibit allergen-specific Th17 cells. Synthetic compounds triggering endosomal TLR on dendritic cells (DC) have been recently proposed as novel adjuvants to improve AIT (Maggi, 2010; Senti et al., 2008). However, the soluble ligands of endosomal TLR have been described to expand *in vivo* Th17 response, to favour autoimmune mechanisms by inducing autoantibodies (Linhart and Valenta, 2012; Akira, 2011). The binding of allergens with this group of adjuvants improves the activity of the constructs, allowing the delivery of the two components inside the same antigen presenting cell (APC) with the outgrowth of innate response able to redirect locally the Th2 response (Matesic et al., 2012; Krieg and Vollmer, 2007). However, no data are known on the activity of these constructs on Th17 cells or on their potential ability to induce a Th17-driven autoimmune response.

We have previously shown that the mixture of an antigen with soluble 2-, 9-substituted 8-OH adenines influenced the profile of

Abbreviations: AIT, allergy immunotherapy; BALF, bronchoalveolar lavage fluid; BMDC, bone marrow dendritic cell; DC, dendritic cell; i.t., intratracheally; MNC, mononuclear cells; nDer p2, natural dermatophagoides pteronyssinus group 2 major allerg.

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T effector cells both in humans and mice (Rappuoli et al., 2011; Kastenmüller et al., 2011). The conjugate (nDer p2-Conj) between the modified adenine SA26E, signaling through human and murine TLR7, and the natural *Dermatophagoides Pteronyssinus* 2 (nDer p2) allergen, was able to redirect nDer p2-specific Th2 responses *in vitro* and to prevent the development of airway inflammation *in vivo* in a short term protocol (Fili et al., 2013). Moreover, when administered in allergen-sensitized mice in a therapeutic setting, both nDer p2- and ovalbumin (OVA)-Conj improved airway inflammation and redirected Th2 effector cells into IL-10- and IFN- γ -producing T cells associated with reduced IgE and increased IgG2a antibodies (Nencini et al., 2015).

The aim of this study was to evaluate the ability of nDer p2-Conj to elicit *in vivo* Th17 response, especially when assayed in long-term models. We provided evidence that the efficacy of the treatment with these compounds on airway inflammation is maintained for more than one year by using a priming protocol. More importantly, even though the nDer p2-Conj has the potential to elicit Th17 cells, its ability to strongly stimulate IL-10 fully inhibits development and expansion of Th17 cells. Thus, the nDer p2-Conj must be considered a valid candidate for novel formulations of AIT due to their prolonged effects and the relative safety.

2. Materials and methods

2.1. Reagents

The LoToxTM nDer p2 used throughout the study was purchased from Indoor Biotechnologies Ltd. (batch n. 31059) (Charlottesville, US) and certified to contain less than <0.03 EU/ μ g endotoxin. OVA was purchased from Invivogen (Milan, Italy). Low-endotoxin RPMI 1640 medium (VLE-RPMI 1640, Biochrom AG, 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 (FCS) was from HyClone (Thermo Scientific, Milan, Italy). Phorbol 12-myristate 13-acetate (PMA) and ionomycin (I) were purchased from Sigma-Aldrich (Milan, Italy). The endotoxin content of all the final reagents (allergens, SA26E and their conjugates) before use was assessed by LAL Test (BioWhittaker) which resulted consistently lower than the detection limit of the assay. Anti-murine CD3 (PE), CD4 (APC-FITC) and CD19 (APC) mAbs were purchased from Miltenyi Biotech (Bergisch Gladbach, Germany). Anti-Mouse Ig, λ 1, λ 2 and λ 3 Light Chain FITC and Anti-Mouse Kappa PE were purchased from Becton-Dickinson (Mountain View, CA).

2.2. Mice

Female IL-10-deficient mice (B6.129P2-*Il10tm1Cgn/J*; stock #002251) and female 5-week-old NOD/LtJ were obtained from the Jackson Laboratory (Bar Harbor, ME). Female 6- to 8-week-old 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.

2.3. Synthesis of SA26E and preparation of allergen-Conj

SA26E synthesis and its binding to nDer p2 or OVA were performed as previously described (Fili et al., 2013; Nencini et al., 2015). Briefly, SA26E was dissolved in 130 μ l DMSO (Sigma Aldrich, Milan, Italy) and chemically conjugated with 3 mg of purified OVA or 1.5 mg of nDer p2 in phosphate buffer (to final volumes of 1.5 and 3 ml, respectively) by overnight incubation at 4°C with continuous rotation. Unconjugated SA26E was then removed by repeated dialysis (2000 mw cut-off, Slide-A-Lyzer cassettes, Pierce, Rockford, IL,

USA) with PBS. Conjugates were aliquoted and stored at –20°C until use.

2.4. In vivo protocols

2.4.1. Short-term priming and therapeutic model of murine airway inflammation

Mice were given the i.p. injection of alum-absorbed nDer p2 (10 μ g) or nDer p2-Conj twice, followed by two intratracheal (i.t.) challenges with allergen (10 μ g in 50 μ l of PBS) on 14d and 18d. Three days after the last challenge the analysis was performed (Fig. 1A).

In the therapeutic protocol, mice were sensitized (i.p.) and challenged (i.t.) twice with nDer (10 μ g). Treatment consisted of administration of nDer p2-Conj (i.p.) at 21d, 23d, 26d and 28d. After the last administration of the adduct, mice were i.t. re-challenged with allergen on 49d and 53d and sacrificed three days later for analysis (Fig. 1B).

2.4.2. Long-term priming and therapeutic model of murine airway inflammation

Mice were sensitized and challenged as above described. To evaluate the long-term effect of immunization with Der p2- (or OVA-) Conj, mice were bred and maintained in pathogen-free condition for 12 months after the second i.t. challenge. After one year, i.t. challenge was repeated twice and three days later animals were sacrificed (Fig. 1C). Moreover, to evaluate the therapeutic effect in a long-term protocol, the group of nDer p2-sensitized mice were ip.-treated at 330d with allergen or nDer p2-Conj in a therapeutic setting, and analysed at 365d after sensitization (Fig. 1D).

nDer p2 or nDer p2-Conj (10 μ g) were administered *via* i.p. in NOD/LtJ mice and the glycemia level was monitored weekly (Fig. 4A).

2.5. Evaluation of airway hyperresponsiveness and bronchoalveolar lavage

Airway hyperresponsiveness (AHR) in response to increasing doses of inhaled methacholine (Sigma-Aldrich) was measured as described (Hoymann, 2007).

Bronchoalveolar lavage (BAL) was performed and analysed as previously described (Vultaggio et al., 2011).

2.6. Lung histology

Lung sections were stained with haematoxylin and eosin (H&E) and periodic acid Schiff (PAS) to evaluate lung inflammation and goblet cell hyperplasia according to scores previously described (Fili et al., 2013; Vultaggio et al., 2011).

2.7. Generation of BMDC

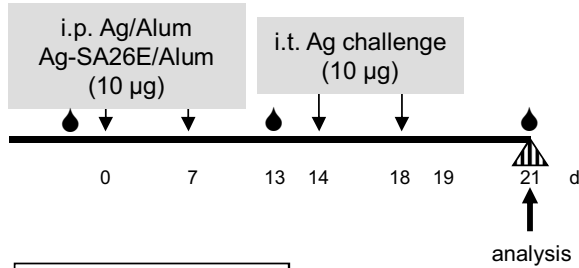
BMDC were prepared according to well-defined protocols (Vultaggio et al., 2009) and 1×10^6 cells were cultured *in vitro* at d8 for 6 h (mRNA detection) and 72 h (protein detection) with nDer p2 (10 μ g/ml) or nDer p2-Conj (10 μ g/ml), or medium alone. At the end of the cultures, the cells were collected for total RNA extraction and supernatants were assayed for their IL-1 β , IL-23, TGF- β , IL-6, IL-10, IL-27, CXCL10 content.

2.8. Cell isolation and cultures

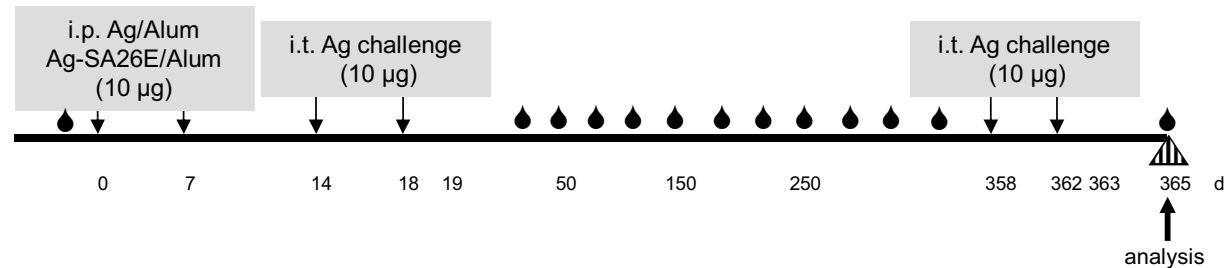
B cells were purified from spleens of wild-type and treated mice by positive selection with anti-CD19 mAb bound to Microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The enrichment of cells examined by

PRIMING PROTOCOLS

A Short-term model



B Long-term model



THERAPEUTIC PROTOCOLS

C Short-term model



D Long-term model

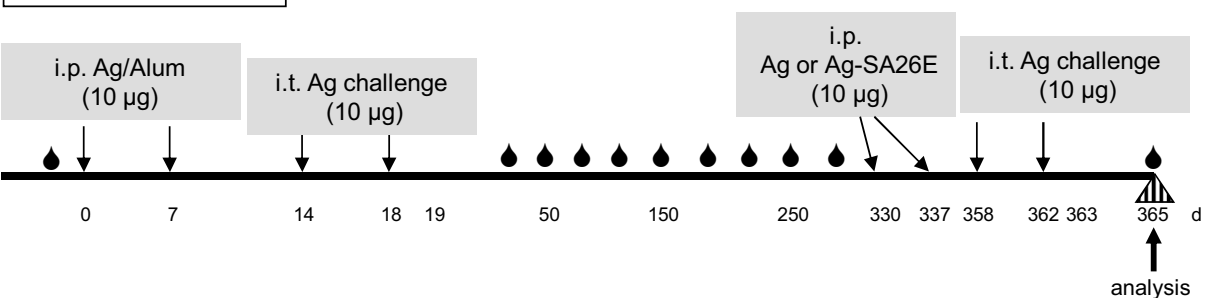


Fig. 1. Experimental protocols.

Time schedule of sensitization, challenges, conjugate administration and analysis in protocols for murine models of experimental airway inflammation. (A–B) Short and long term priming models. (C–D) Short and long-term therapeutic models. Ag: nDer p2 allergen or OVA; Ag-SA26E: Der p2-Conj or OVA-Conj; i.p.: intraperitoneal; i.t.: intratracheal; ●: blood sampling.

cytometry was consistently >95%. B cells were stimulated with medium or conjugate (or allergen alone) at the final concentration of 10 µg/ml for 72 h.

Cytometric analysis of CD19+ cells expressing membrane light chains were also evaluated on unstimulated cells.

2.9. ELISA assays

Lung, spleen and mediastinal lymph nodes mononuclear cells (MNC) were prepared and cultured with allergen as previously described (Vultaggio et al., 2011). Three-day culture supernatants were used for evaluating IL-17A, IFN-γ, IL-10 and IL-13 mea-

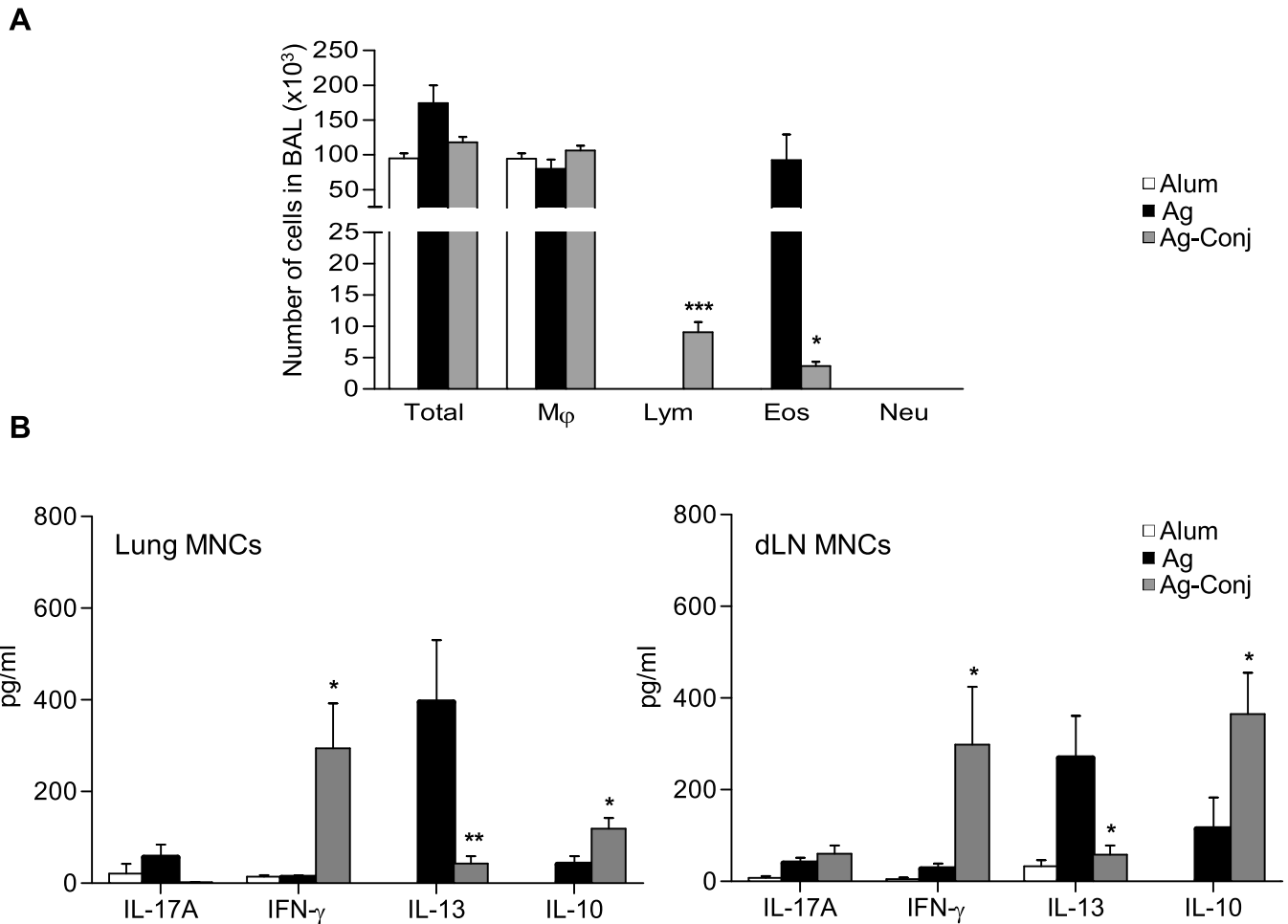


Fig. 2. The priming with nDer p2-Conj did not induce the Th17 response in a short-term setting.

(A) Differential cell counts in BALF performed 72 h after the last challenge. Mφ: macrophages; Lym: lymphocytes; Eos: eosinophils; Neu: neutrophils. (B) Cytokines production upon *in vitro* nDer p2 stimulation (10 μg/ml for 72 h) of lung and draining lymph nodes MNC from C57Bl/6 mice sensitized and challenged as described in Fig. 1A. All reported data are referred to pooled results from three separate experiments; six mice/group/experiment. Data are expressed as the mean values (± SEM) and statistical significance between the nDer p2 and nDer p2-Conj groups is reported. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

sured by commercial ELISA kits (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. Detection limits for IL-17A, IFN-γ, IL-10 and IL-13 were 15 pg/ml, 2 pg/ml, 4 pg/ml and 1.5 pg/ml, respectively. Cytokines produced by Conj-stimulated BMDC were assayed by commercially available ELISA kits (R&D Systems). Detection limits of IL-27, IL-1β, IL-23, IL-6, TGF-β and CXCL10 kits were 19 pg/ml, 7.5 pg/ml, 4.8 pg/ml, 1.8 pg/ml, 15.4 pg/ml, 4.2 pg/ml, respectively.

Total IgE and allergen-specific IgG2a antibodies were measured by ELISA as described (Nencini et al., 2015).

2.10. Quantitative mRNA Analysis

Total lung RNA from snap-frozen mouse lungs were extracted using TRIzol reagent (RNAwiz, Invitrogen, Milan, Italy), whereas total RNA from MNC were extracted using the RNeasy mini kit (Qiagen, Milan, Italy) as previously described (Fili et al., 2013). 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.

2.11. Detection of autoantibodies and tissue biochemical analytes

Serum samples were evaluated for anti-dsDNA, anti-nRNP, anti-Histones, anti-Nuclear autoantibodies and Rheumatoid Factor by dedicated ELISA kits (Alpha Diagnostic International, San Antonio, Texas, USA) according to the manufacturer's instructions.

Serum levels of GPT (Glutamic Pyruvic Transaminase), glucose and urea were determined with Reflotron Plus System (Roche Diagnostics S.p.A, Milan, Italy).

2.12 Statistical Analysis

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

3. Results

3.1. nDer p2-Conj induced increased gene expression of th17-polarizing cytokines in DC

Initially we evaluated the ability of nDer p2-Conj to induce Th17-polarizing molecules by APC *in vitro*. BMDC generated from wild-type mice were cultured with 10 μg/ml of nDer p2-Conj (or the free allergen) and a panel of molecules was detected at the mRNA and protein levels. We observed significantly higher levels

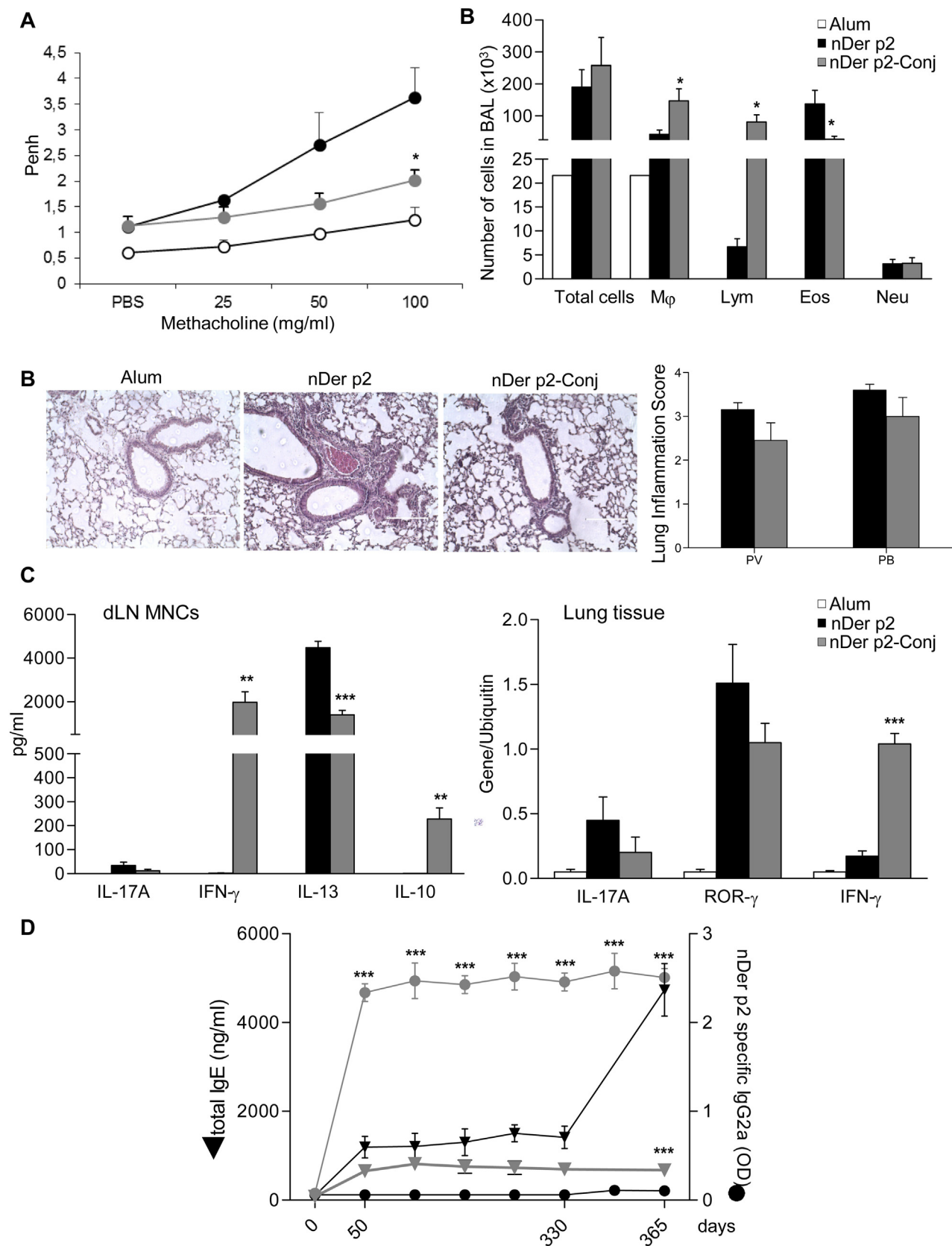


Fig. 3. Effects of nDer p2-Conj in priming long-term protocol.

(A) Airway hyper-reactivity (AHR) was measured 24 h after the i.t. administration of nDer p2 (10 μ g/ml). Penh = Enhanced pause. (B) Differential cell counts in BALF performed 72 h after the last challenge. M ϕ : macrophages; Lym: lymphocytes; Eos: eosinophils; Neu: neutrophils. (C) Histology of lung sections stained with H&E (20 $\times$ magnification) (left panel) and lung inflammation scores (PV, perivascular; PB, peribronchial) (right panel). (D) Cytokine production from dLN MNC upon *in vitro* nDer p2 stimulation (10 μ g/ml for 72 h) (left panel) and mRNA expression in lung tissue (right panel) from C57Bl/6 mice sensitized and challenged as described in Fig. 1B. (E) Longitudinal monitoring of total IgE serum levels (ng/ml) and allergen-specific IgG2a (dilution 1:20) in nDer p2-Conj-primed mice. All reported data are referred to pooled results from two separate experiments; six mice/group/experiment. Data are expressed as the mean values ($\pm$ SEM) and statistical significance between the nDer p2 and nDer p2-Conj groups is reported. * p < 0.05, ** p < 0.01, *** p < 0.001.

Table 1

Cytokines mRNA expression and production by BMDC from wt mice.

	IL-1 β	IL-23	TGF β	IL-6
Gene/Ubiquitin				
nDer p2	0.56 $\pm$ 0.17	0.2 $\pm$ 0.08	5.5 $\pm$ 0.82	0.2 $\pm$ 0.08
nDer p2-conj	5.66 $\pm$ 1.47*	1.54 $\pm$ 0.26**	6 $\pm$ 1.63	4.5 $\pm$ 0.41*
Pg/ml				
nDer p2	20 $\pm$ 7.8	0.1 $\pm$ 0.01	90 $\pm$ 10	350 $\pm$ 37
nDer p2-Conj	144 $\pm$ 10***	125 $\pm$ 25*	66 $\pm$ 14	1339 $\pm$ 83**
	IL-10	IL-27	CXCL10	
Gene/Ubiquitin				
nDer p2	0.42 $\pm$ 0.12	0.36 $\pm$ 0.03	1 $\pm$ 0.41	
nDer p2-conj	4.3 $\pm$ 0.36***	6.1 $\pm$ 1.05**	16.5 $\pm$ 2.86*	
Pg/ml				
nDer p2	7.2 $\pm$ 4.9	5.4 $\pm$ 4.3	167 $\pm$ 27	
nDer p2-Conj	334 $\pm$ 214	220 $\pm$ 75*	1000 $\pm$ 501	

Cytokines mRNA expression was evaluated by real-time quantitative PCR. Cytokines production was measured by ELISA commercial kits. Data are expressed as mean values ($\pm$ SEM) of gene/ubiquitin ratios or of proteins in culture supernatants. Data are expressed as a difference compared to the medium by itself.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

of cytokines, able to develop and polarize (IL-1 β , IL-6,) or amplify (IL-23) Th17 cells, with the nDer p2-Conj than with the free allergen (Table 1). By contrast, no increase in TGF- β was observed. Furthermore, significantly higher levels of the IL-1 β , IL-6 and IL-23 were also induced by the nDer p2-Conj than nDer p2 when cultured with purified CD11c $^{+}$ spleen cells from wt C57BL/6 mice (data not shown). We also confirmed the induction of higher levels of pro-inflammatory (IL-27, CXCL10) and regulatory (IL-10) cytokines by the nDer p2-Conj than the corresponding free allergen (Filì et al., 2013; Nencini et al., 2015).

3.2. The allergen-specific th17 response was not increased in nDer p2-Conj-primed mice

We then performed experiments to investigate whether Th17 cells were induced *in vivo* by the nDer p2-Conj in murine models of airway inflammation by using short-term priming and therapeutic protocols of administration (Fig. 1A and B).

nDer p2-Conj-primed mice showed a lower proportion of eosinophils and a higher number of lymphocytes in BALF than controls, with no change of neutrophils (Fig. 2A). Allergen-stimulated MNC obtained from lung and draining lymph nodes of nDer p2-Conj-primed mice produced a higher amount of IFN- γ and IL-10 and lower levels of IL-13 than control mice. More importantly, IL-17A was not increased in nDer p2-Conj treated mice compared to controls (Fig. 2B).

3.3. The nDer p2-Conj did not stimulate IL-17A or B cell expansion and autoantibodies production, in a long-term priming model

To evaluate whether the effects of the nDer p2-Conj were maintained for a long time, we analysed mice 12 months after the sensitization with nDer p2-Conj according to the protocol showed in Fig. 1B. Twenty-four hours after the i.t. challenge with allergen, AHR was significantly reduced in nDer p2-Conj sensitized mice (Fig. 3A). These mice displayed a significant decrease of eosinophils and a significant increase in lymphocytes in BALF with no change of neutrophils (Fig. 3B). Moreover, the histochemical analysis showed the reduction of cellular infiltrates in perivascular and peribronchial lung compartments (Fig. 3C), without change in the mean proportion ($\pm$ SEM) of PAS-positive epithelial cells (PAS scores 0.82 ± 0.01 vs 0.80 ± 0.1) in nDer p2-Conj-sensitized mice compared to controls. Of note, there was a clear increase in IL-10 and IFN- γ and a decrease of IL-13 by allergen-stimulated lymph node MNC, while the IL17A levels did not change (Fig. 3D, left panel), in addition ROR- γ t and IL-17A were similarly expressed in lung tissue from nDer p2-Conj-treated-mice as in the controls (Fig. 3D, right panel). We also observed a significant reduction of total serum IgE paralleled by the increase in allergen-specific IgG2a (Fig. 3E). Finally, Foxp3 mRNA was similarly expressed in the allergen-stimulated MNC from lymph nodes of nDer p2-Conj- (0.11 ± 0.04) and nDer p2- (0.08 ± 0.03) treated-mice.

To evaluate the activity of another allergen, we used OVA or OVA bound to the SA26E. By using the priming long-term protocol (Fig. 1B), we obtained similar results of nDer p2 model such as a significant decrease of eosinophils in BALF with no change of neutrophils, a significant increase in IFN- γ and IL-10 by OVA-

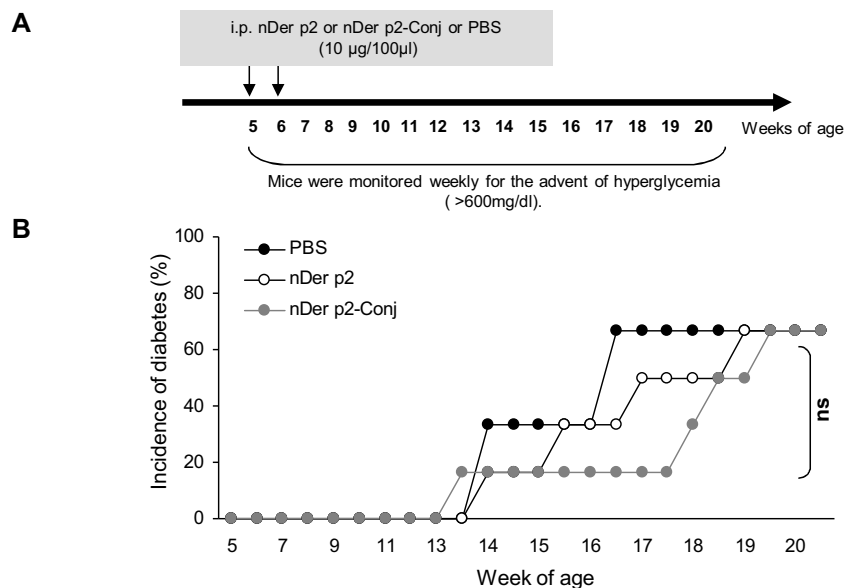


Fig. 4. The conjugate priming did not influence the onset of diabetes in non-obese NOD/LtJ mice.

(A) Time schedule of conjugate administration and blood sampling in NOD/LtJ mice. (B) Incidence of diabetes (%) in controls (PBS-treated, age-matched) and in nDer p2 and nDer p2-Conj-treated NOD mice as a function of age. All reported data are referred to pooled results from two separate experiments; six mice/group/experiment.

Table 2
Serum levels of autoantibodies in long-term primed mice.

Group of mice	ANA	Anti ds DNA Abs	Anti Histone Abs	Anti nRNP Abs	RF
Alum	0.33 ± 0.01	1.62 ± 0.15	0.88 ± 0.21	0.33 ± 0.01	0.24 ± 0.03
nDer p2	0.31 ± 0.02	1.71 ± 0.27	0.76 ± 0.22	0.46 ± 0.22	0.29 ± 0.03
nDer p2-Conj	0.30 ± 0.02	1.07 ± 0.015	0.53 ± 0.18	0.28 ± 0.01	0.33 ± 0.006

Autoantibodies were measured by ELISA commercial kits. OD ± SEM are reported.

ANA: antinuclear antibody; dsDNA: double stranded DNA; nRNP: nuclear ribonucleoprotein; RF: rheumatoid factor.

P=NS among different groups of mice.

stimulated lung MNC and a significant increase in OVA-specific IgG2a in OVA-Conj sensitized mice (Fig. A1).

The long-term model was then used to evaluate the potential of the conjugate to promote the onset of autoreactivity and B cell expansion. No increase in serum levels of autoantibodies (ANA, anti-dsDNA, anti-Histones, anti-nRNP Abs and Rheumatoid factors) was found in the nDer p2-Conj-treated mice compared to controls (Table 2). Noteworthy, in these animals the size and weight of mesenteric, mediastinal lymph nodes or spleens were similar to those in untreated mice (data not shown). No differences were detected in the proportions of CD19+ splenic cells and the κ/λ chain usage. In addition, their *in vitro* proliferation and IL-10 production to polyclonal T-independent stimuli, as resiquimod (R848), were unchanged in nDer p2-Conj- vs allergen-primed mice (Fig. A2 and data not shown). Moreover, serum GPT, glycemia and urea, found in nDer p2-Conj-primed mice resulted in the normal range (Table A1).

Lastly, we evaluated the potential pro-diabetogenic activity of the nDer p2-Conj in NOD/LtJ mice that spontaneously develop autoimmune diabetes (Fig. 4A). No significant difference in the timing of the onset of stable hyperglycemia was observed in nDer p2-Conj- vs nDer p2-primed NOD/LtJ mice (Fig. 4B).

3.4. The allergen-specific *th17* response was not increased in nDer p2-Conj-treated mice

When the therapeutic setting was used (Fig. 1C), the nDer p2-Conj-treated mice, were characterized by a reduction (even if not significant) of eosinophils, an increase in lymphocytes and a significant reduction of neutrophils in BALF (Fig. 5A). At protein levels, lung MNC of nDer p2-Conj-treated mice produced a higher amount of IFN- γ and IL-10 and lower levels of IL-13 than control mice, without any increase in IL-17A production (Fig. 5B). Similarly, ROR- γ t and IL-17A mRNA expression in allergen-stimulated lung MNC and draining lymph nodes was not increased (Fig. 5C).

To better mimic the human disease, we evaluated whether the nDer p2-Conj was also effective in long-term sensitized animals analyzing the impact of nDer p2-Conj treatment on the airway inflammation in mice sensitized with nDer p2 one year before (Fig. 1D).

Twenty-four hours after the i.t. challenge with allergen, AHR was significantly reduced in nDer p2-Conj treated mice (Fig. 6A). In these mice we observed the reduction of eosinophils and neutrophils in BALF (Fig. 6B), and the histochemical analysis showed a significant reduction of cellular infiltrates in perivascular and peribronchial lung compartments (Fig. 6C), with a decreased mean proportion ($\pm$ SEM) in PAS-positive epithelial cells (PAS scores 0.25 ± 0.14 vs 0.80 ± 0.1 , $p < 0.05$) in nDer p2-Conj-sensitized mice compared to controls. Moreover, we observed the increased production of IL-10 and IFN- γ by nDer p2-stimulated MNC from draining lymph nodes (Fig. 6D). ROR- γ t (at the mRNA level) and IL-17A (at the mRNA and at the protein levels) were not increased in lung tissue or in nDer p2-stimulated lymph nodes MNC from nDer p2-Conj-treated-mice (Fig. 6D and data not shown). The reduction

of total IgE and the increase in allergen-specific IgG2a in the serum was also observed (Fig. 6E).

In agreement with these data, the increase in autoantibodies and B cell expansion was not observed in the long-term treated mice (data not shown).

3.5. nDer p2-Conj-driven IL-10 inhibited the allergen-specific *th17* response

Finally, we questioned ourselves whether the nDer p2-Conj-induced IL-10 could be responsible for the lack of induction of IL-17A production. Thus, we performed experiments with the nDer p2-Conj in IL-10KO mice by using the short-term therapeutic protocol previously described (Fig. 1B). In BALF from IL-10KO mice, the Der p2-Conj induced a significant increase in neutrophils compared to nDer p2-treated IL-10KO mice (Fig. 7A). Accordingly, the cellular infiltrates in perivascular and peribronchial lung compartments were increased in nDer p2-Conj-treated mice compared to controls (Fig. 7B). The ROR- γ t and IL-17A mRNA expression was highly increased in allergen-stimulated MNC from draining lymph nodes of nDer p2-Conj-treated IL-10KO mice (Fig. 7C). IL-17A levels were up-regulated in allergen-stimulated MNC derived from the lungs and spleens from the same group of mice (Fig. 7D). The high levels of IL-17A were always associated with the increase in other type 1 and type 2 cytokines. (Fig. 7D). Accordingly, the mean values of OD ($\pm$ SEM) of ANA autoantibodies were significantly ($p = 0.03$) increased in nDer p2-Conj- (0.74 ± 0.05) compared to nDer p2- (0.54 ± 0.04) treated mice.

4. Discussion

An efficacious approach to treating allergy is represented by AIT, which is able to modify the pathogenetic allergen-specific Th2-responses (Maggi et al., 2012). We previously showed that the stable binding of nDer p2 with an 8-OH-modified adenine signaling through TLR7, called SA26E, improved vaccine efficacy leading to a more effective modulation of allergen-specific Th2 cell response (Fili et al., 2013). However, some recent reports, indicated that the ligands of some endosomal TLR, such as TLR7 and TLR9, promote the onset of autoimmunity *in vivo* (Avalos et al., 2010).

In this paper, we investigated whether our conjugate develops and amplifies the Th17 response *in vivo*, a profile usually associated with autoimmunity, in both short- and long-term protocols of priming and treatment of murine airway inflammation. We showed that the conjugate was able to elicit *in vitro* production of cytokines responsible for the polarization and amplification of Th17 response, such as IL-6, IL-1 β and IL-23, thus suggesting its potential role in favouring the onset of autoimmunity. However, when used *in vivo* in a short-term priming model of airway inflammation, the nDer p2-Conj induced the shift of allergen-specific Th2 response to more protective IFN- γ and IL-10 profiles, without any increase of IL-17A production. Additionally, our data showed that also in a therapeutic short-term model the nDer p2-Conj induced the shift of allergen-specific Th2 cells in the lung to T cells producing IFN- γ and IL-10, without any increase in ROR- γ and IL-17A mRNA expression.

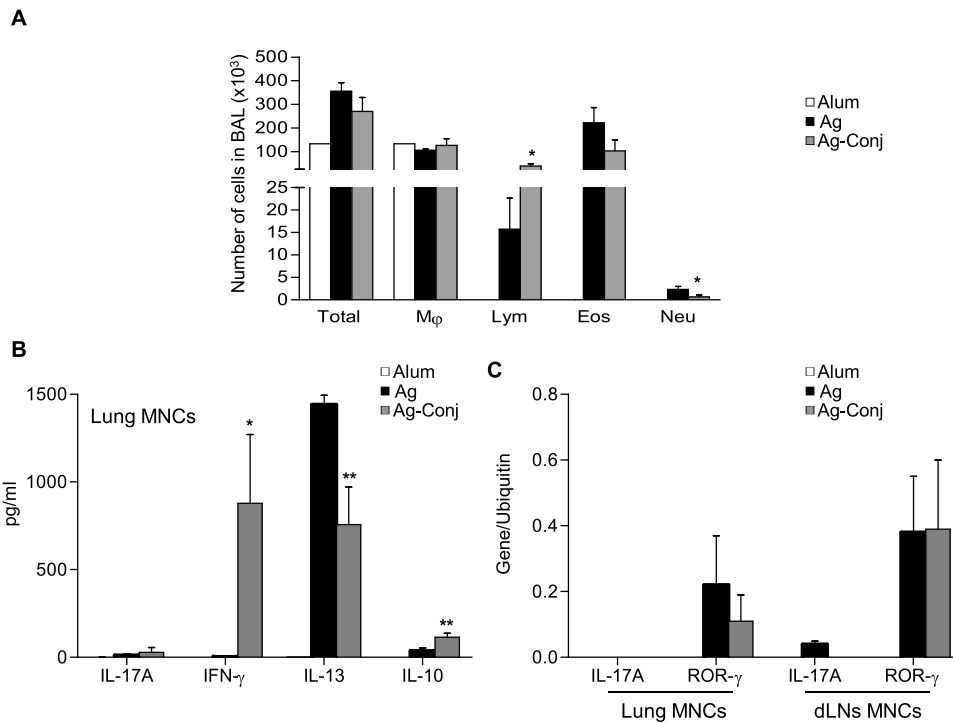


Fig. 5. The nDer p2-Conj did not influence the Th17 response in short-term therapeutic setting.

(A) Differential cell counts in BALF performed 72 h after the last challenge. Mφ: macrophages; Lym: lymphocytes; Eos: eosinophils; Neu: neutrophils. (B) Cytokines production upon *in vitro* nDer p2 stimulation (10 μg/ml for 72 h) of lung MNC from C57Bl/6 mice sensitized and challenged as described in Fig. 1C. (C) mRNA expression of IL-17A and ROR-γ in lung and lymph nodes MNC from the same mice. All reported data are referred to pooled results from three separate experiments; six mice/group/experiment. Data are expressed as the mean values (± SEM) and statistical significance between the nDer p2 and nDer p2-Conj groups is reported. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

The *in vitro* and *in vivo* results appear to be conflicting, but they suggest that, even though the activation of BMDC leads to the production of pro-Th17 cytokines, the parallel upregulation of IL-10 by the same cells *in vitro* and *in vivo* may deeply interfere with the development of Th17 effectors.

More importantly, we provided evidence that the conjugate was also efficacious when administered to animals that had been sensitized the year before, a condition mimicking the AIT treatment in human allergy. In this model, the decrease of eosinophils was associated with no change in the proportion of neutrophils in BALF. By using the priming protocol, we confirmed that the effects of nDer p2-Conj on airway inflammation were maintained for more than one year. In this model, we observed the decrease of eosinophils in BALF, the increase in IL-10 and IFN-γ in cultures of allergen-stimulated lymph node MNC and the sustained IgG2a levels paralleled by the decrease of serum IgE. These alterations were not associated with any increase in neutrophils in BALF and in IL-17A and ROR-γ in the lungs of nDer p2-Conj-treated mice. Of note, similar results were obtained with another conjugate (OVA bound with the 8-OH modified adenine SAE2) by using the same long-term protocol.

Consequently, to establish the role of regulatory cytokines in our models, we performed *in vivo* experiments with the nDer p2-Conj in IL-10KO mice. The increase in neutrophils in BALF and the up-regulation of ROR-γ and IL-17A mRNA expression in allergen-stimulated lung and lymph node MNC was clearly shown. Importantly, the parallel increase in other adaptive cytokines in these experiments reinforced the concept that IL-10 stimulated *in vivo* by the conjugate plays a vigorous regulatory role in the activation of the entire memory T cell compartment, including Th17.

These data strongly suggest that in wild type mice the conjugate-driven IL-10 currently dampens all adaptive cytokines

including type 2 molecules, contributing solely to the modulation of allergic airway inflammation.

In addition, they also suggest a particular attention to the use of conjugates in patients who have dysregulated immune responses (like IL-10KO mice) and who are more susceptible to autoimmune disease. Indeed nDer p2-Conj could increase allergic airway inflammation in people who do not have good IL-10/regulatory responses.

We showed that the nDer p2-Conj was able to induce IL-27 production *in vitro*, known to inhibit Th17 cells in both an IL-10-dependent and an independent manner (Pot et al., 2011; Touzot et al., 2015). At the moment, we cannot rule out an IL-27-mediated, IL-10 independent mechanism in our models. The involvement of other regulatory mechanisms seems to be unlikely; indeed the nDer p2-Conj was unable to stimulate TGF-β deriving from BMDC and Foxp3 mRNA expression was not up-regulated in tissues or in allergen-stimulated MNC in short- and long-term models of airway inflammation. Overall, these data clearly suggest that the conjugate-driven IL-10 is more able to block IL-17A response in different models of airway inflammation than other mechanisms. From the moment that the nDer p2-Conj was able to induce B cell growth factors such as IL-6 and IL-10 on BMDC, we evaluated the *in vivo* effects of this compound on B cell compartment by using a long-term (one year) priming model. In this setting, we did not provide any evidence of undesired effects related to chronic TLR7 triggering, such as B cell activation and autoimmunity. In the long-term experiments, the treatment with the conjugate did not change either the number, the k/λ light chain usage and the proliferation rate of purified spleen B cells, or IL-10 production by these cells. This result confirmed the integrity of B cell compartment in lymphoid organs and excluded monoclonal lymph proliferative expansion. These data are partially at odds with some reports showing that autoreactive B cells can be activated and induced to produce autoantibodies *via* the signals on

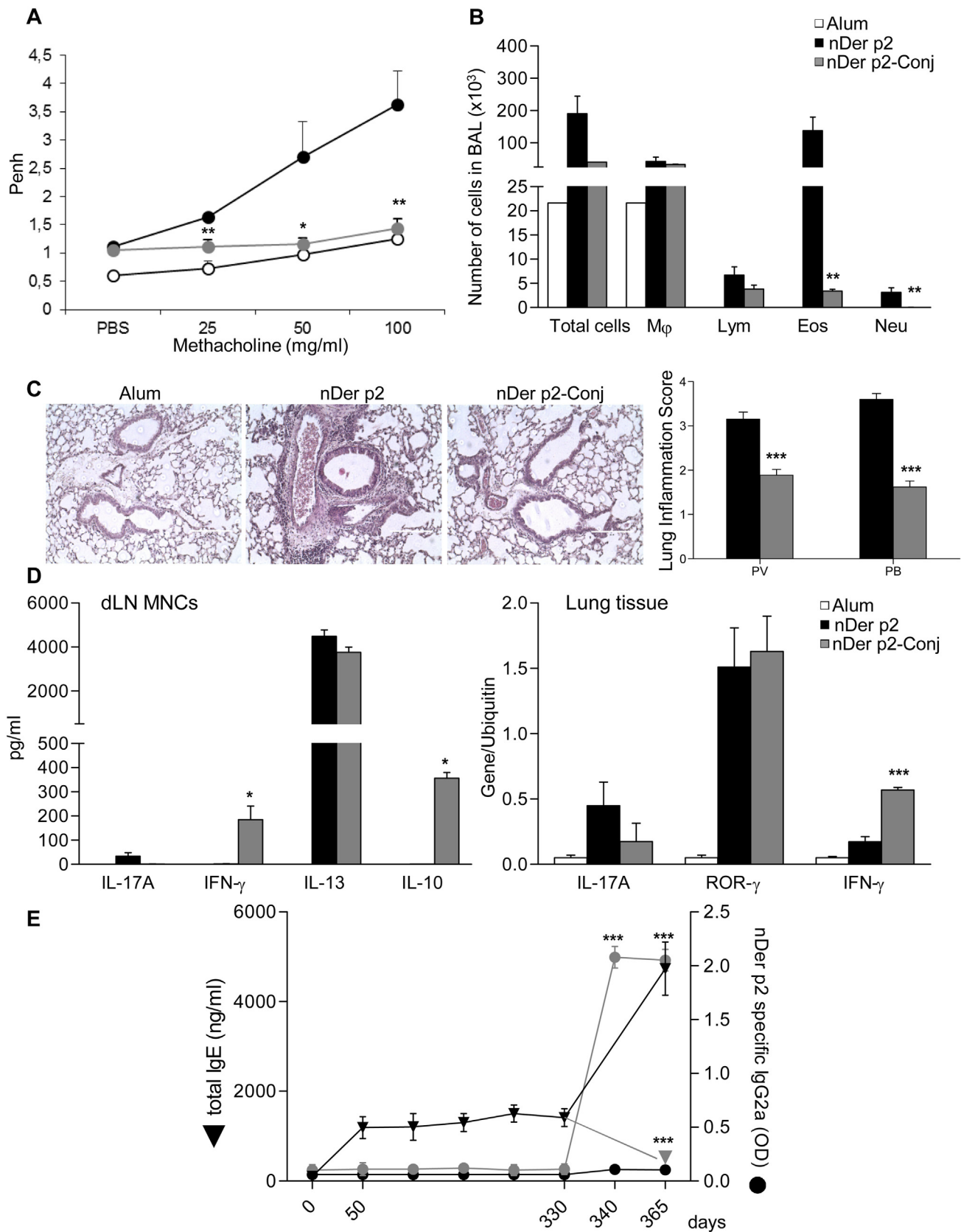


Fig. 6. Therapeutic effects of nDer p2-Conj in long-term nDer p2-sensitized mice.

(A) Airway hyper-reactivity (AHR) was measured 24 h after the i.t. administration of nDer p2 (10 µg/ml). Penh = Enhanced pause. (B) Differential cell counts in BALF performed 72 h after the last challenge. Mφ: macrophages; Lym: lymphocytes; Eos: eosinophils; Neu: neutrophils. (C) Histology of lung sections stained with H&E (20x magnification) (left panel) and lung inflammation scores (PV, perivascular; PB, peribronchial) (right panel). (D) Cytokine production from MNC of draining lymph nodes upon *in vitro* nDer p2 stimulation (10 µg/ml for 72 h) (left panel) and mRNA expression in lung tissue (right panel) from C57Bl/6 mice sensitized and challenged as described in Fig. 1D. (E) Longitudinal monitoring of total IgE serum levels (ng/ml) and allergen-specific IgG2a levels (dilution 1:20) in nDer p2-sensitized mice treated with the conjugate as described in Fig. 1D.

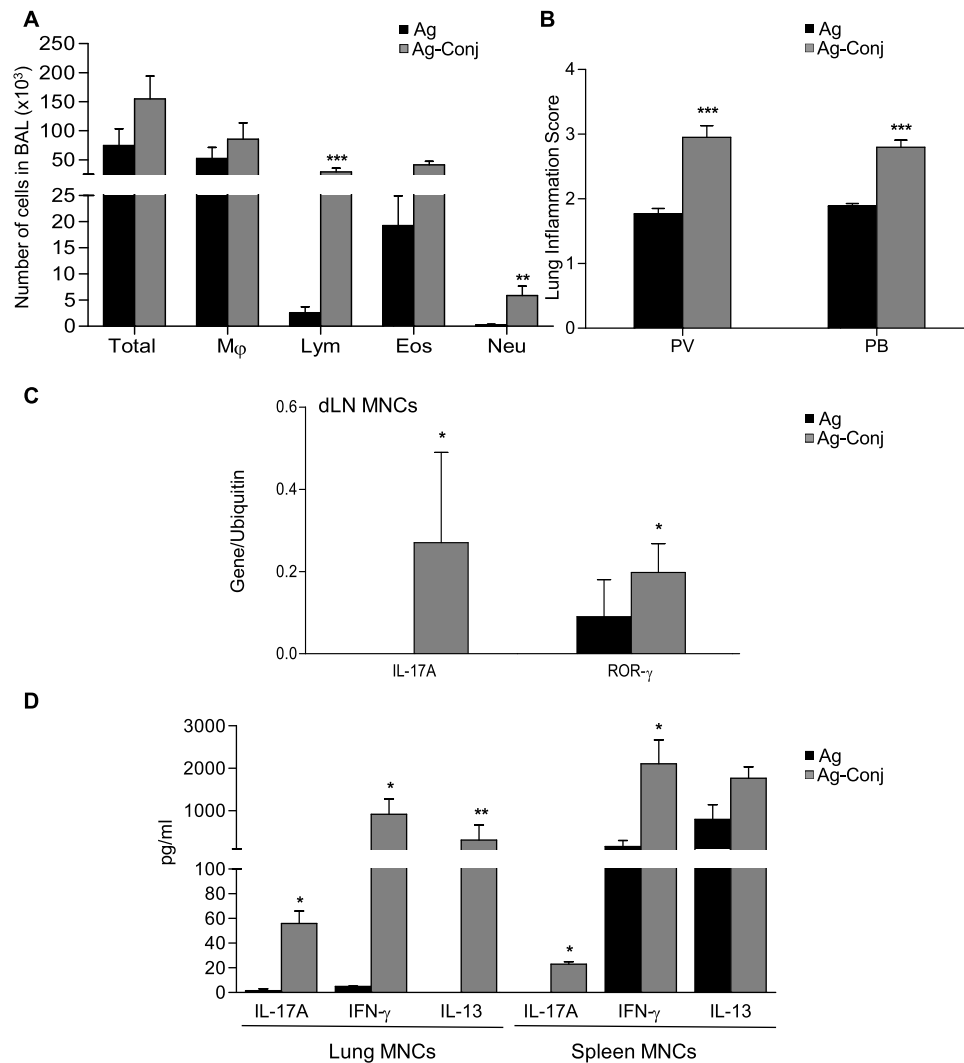


Fig. 7. Effects of nDer p2-Conj on IL-17 production in IL-10KO mice.

(A) Differential cell counts in BALF performed 72 h after the last challenge. Mφ: macrophages; Lym: lymphocytes; Eos: eosinophils; Neu: neutrophils. (B) Lung inflammation scores (PV, perivascular; PB, peribronchial). (C) IL-17 and ROR-γ mRNA expression in allergen-stimulated MNC of draining lymph nodes from IL-10KO mice sensitized and challenged as described in Fig. 1B. (D) Cytokines production upon *in vitro* nDer p2 stimulation (10 μg/ml for 72 h) of MNC of lung and spleen from the same mice. All reported data are referred to pooled results from three separate experiments; six mice/group/experiment. Data are expressed as the mean values (± SEM) and statistical significance between the nDer p2 and nDer p2-Conj groups is reported. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

BCR (autoantigen) and TLR7 (RNA-containing immunocomplexes) (Marshak-Rothstein, 2006). In addition, it has been shown that a TLR7 polymorphism is highly associated with human SLE (Wang et al., 2014; Tian et al., 2012). However, our previous and current data indicate that the conjugate did not show any ability to induce *in vitro* proliferation of human or murine B cells, while to the contrary other endosomal TLR7 or TLR9 ligands such as imidazoquinolines or CpG-ODNs did (Simchoni and Cunningham-Rundles, 2015). It is generally agreed that molecular structure, local amount, affinity for the receptors and time of the interaction may affect the quality and intensity of signals exerted by different ligands of the same TLR agonists: this could explain potential differences between the effects of modified adenines and other TLR7 ligands (Chang, 2010). Finally, the conjugate did not promote the onset of spontaneous hyperglycemia when administered to NOD/LtJ mice, confirming its inability to elicit or amplify autoimmune response and related disorders.

Although further experiments are needed to establish a definitive safety profile, our *in vitro* and *in vivo* findings suggest that the conjugate between the allergen and 8-OH-modified adenine redirects Th2 response to a balanced Th1/Tr1 profile without increasing Th17 response and inducing autoimmunity.

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Conflict of interest

E.G.O., S.R., E.M. and P.P. have a patent with the University of Florence and Azienda Ospedaliera –Universitaria Careggi. The other authors declare no conflicting interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.molimm.2016.07.011>.

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Table A1. Serum levels of GTP, glycemia and urea in long-term primed mice

Group of mice	GPT (U/l)	GLYCEMIA (mg/dl)	UREA (mg/dl)
Alum	8 ± 1	179 ± 20	63 ± 2
nDer p2	8 ± 0.5	129 ± 1	50 ± 1.3
nDer p2-Conj	7 ± 1.7	129 ± 22	36 ± 3.2

Serum levels were determined by Reflotron Plus System.

GPT: glutamic pyruvic transaminase

Range for: GPT<40 U/l; GLYCEMIA 70-110 mg/dl; UREA <50<71 mg/dl

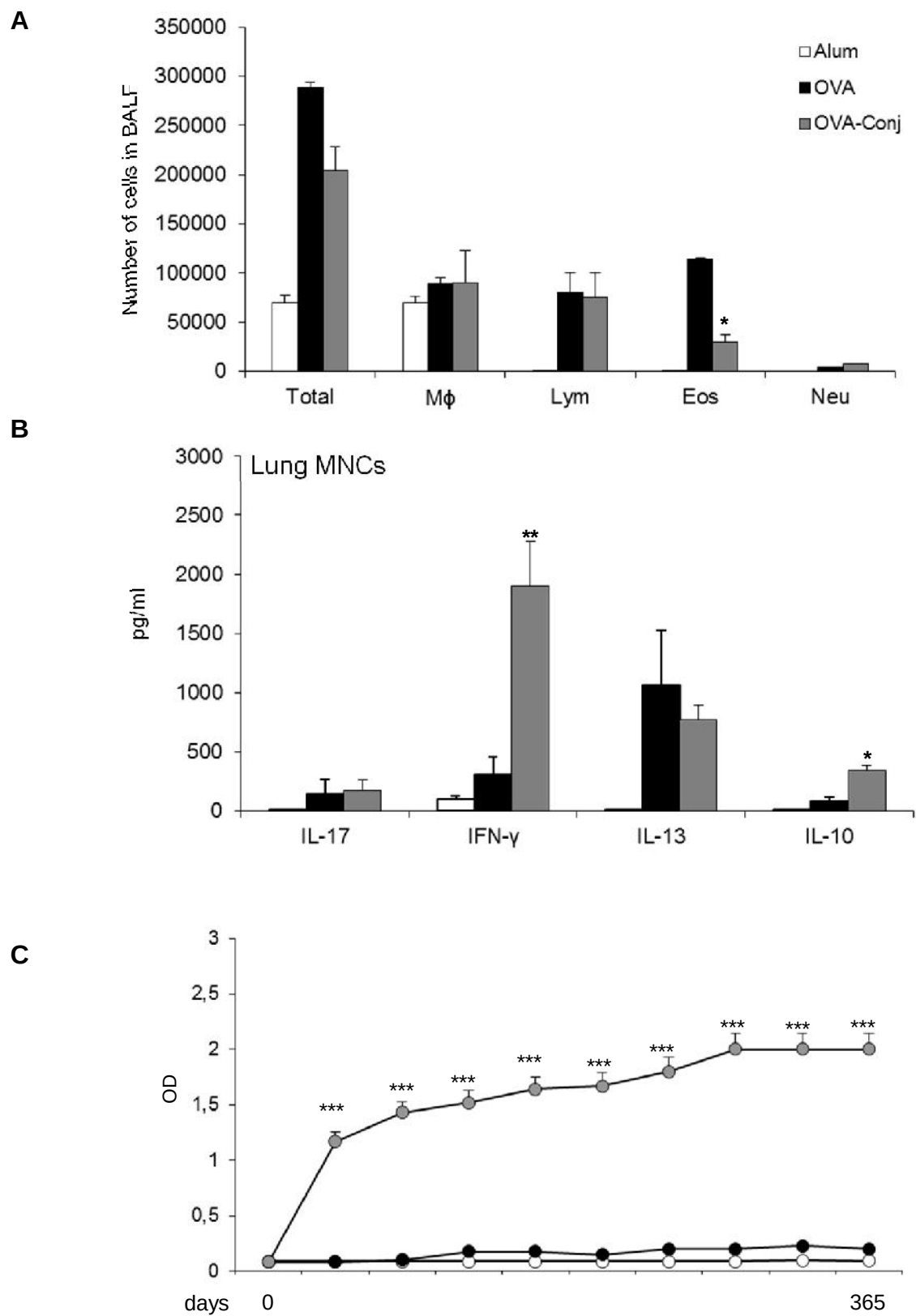


Figure A1

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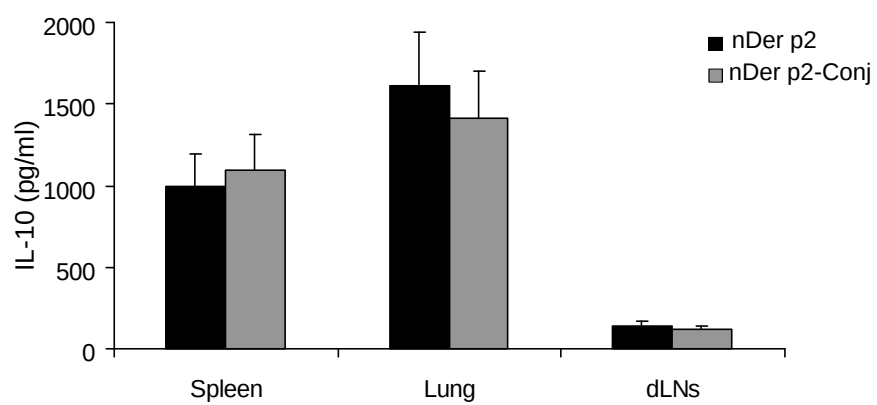


Fig. A2