Molecular Immunology 77 (2016) 60–70

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

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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ARTICLE INFO

Article history:
Received 17 May 2016
Received in revised form 12 July 2016
Accepted 14 July 2016
Available online 29 July 2016

Keywords:
Adjuvants
Vaccines
Toll-like receptors
Adenine-derivatives
Immunotherapy

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-γt 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.

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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http://dx.doi.org/10.1016/j.molimm.2016.07.011
0161-5890/

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 (Filì 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 LoTox™ 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/μg 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 μg/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, λ1, λ2 and λ3 Light Chain FITC and Anti-Mouse Kappa PE were purchased from Becton-Dickinson (Montain 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 (Filì et al., 2013; Nencini et al., 2015). Briefly, SA26E was dissolved in 130 μl 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, Slyde-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 μg) or nDer p2-Conj twice, followed by two intratreacheal (i.t.) challenges with allergen (10 μg in 50 μl 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 μg). 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 μg) 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 (Filì 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 μg/ml) or nDer p2-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-1β, 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