



Mucosal expression of DEC-205 targeted allergen alleviates an asthmatic phenotype in mice

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ABSTRACT

Considering the rising incidence of allergic asthma, the symptomatic treatments that are currently applied in most cases are less than ideal. Specific immunotherapy is currently the only treatment that is able to change the course of the disease, but suffers from a long treatment duration. A gene based immunization that elicits the targeting of allergens towards dendritic cells in a steady-state environment might have the potential to amend these difficulties. Here we used a replication deficient adenovirus to induce the mucosal expression of OVA coupled to a single-chain antibody against DEC-205. A single intranasal vaccination was sufficient to mitigate an OVA-dependent asthmatic phenotype in a murine model. Invasive airway measurements demonstrated improved lung function after Ad-Dec-OVA treatment, which was in line with a marked reduction of goblet cell hyperplasia and lung eosinophilia. Furthermore OVA-specific IgE titers and production of type 2 cytokines were significantly reduced. Together, the here presented data demonstrate the feasibility of mucosal expression of DEC-targeted allergens as a treatment of allergic asthma.

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1. Introduction

Asthma is one of the major issues of public health in industrialized countries with an estimated 300 million affected people worldwide and annual costs (both direct and indirect) of 18 billion in the USA alone [1]. Symptomatically, asthma manifests in airway hyper-responsiveness (AHR) towards unspecific stimuli, airway remodeling, and episodes of wheezing and airway obstruction. Asthma can be divided into multiple endotypes with diverse etiologies, leading to the view that asthma is rather a syndrome than a single disease [2]. Nevertheless, most cases of asthma, especially during childhood, are attributed to

allergic reactions [3] towards harmless airborne allergens. This allergy-dependent form of asthma is associated in about 90% of cases with a Th2 polarization of the antigen-specific T-cell response, allergen-specific IgE production and infiltration of eosinophilic granulocytes into the lung after inhalation of the allergen [4]. The standard therapy of asthma consists of glucocorticoids and β -adrenergic agonists, which unfortunately is a purely symptomatic treatment and ineffective in about 5–10% of patients [5]. Additionally, allergies in general are treated by specific immunotherapy (SIT). This treatment consists of increasing doses of the causative allergen given subcutaneously (SCIT) or sublingually (SLIT) [6]. The exact mechanism of SIT is not fully elucidated yet and might vary for different regimens. However, the induction of regulatory T-cells and protective IgG antibodies that block IgE-mediated activation of effector cells are likely to be involved. Although the safety profile as well as the efficacy of SLIT and SCIT were demonstrated in recent reviews [7,8], the major drawbacks of these therapies are the long-treatment-schedule with around 3–5 years and that it is not effective in all allergic individuals. Attempts to reduce IgE mediated side effects of SIT by the use of recombinant mosaic proteins, which lack most of the conformational IgE-epitopes, have led to promising results for some major allergens. However, these mosaic proteins require specific [9], and occasionally extensive [10], molecular adaption for each allergen. Another possible improvement to SIT might be the intralymphatic

Abbreviations: BAL(F), broncho alveolar lavage (fluid); IL, Interleukin; IFN γ , Interferon- γ ; DCs, dendritic cells; Th1/2, T helper type 1/2; sc, single-chain; PerCP, peridinin chlorophyll protein; PE, phyco-erythrin; FACS, fluorescence activated cell sorter; HRP, horse radish peroxidase; ECL, enhanced chemiluminescence; GFP, green fluorescent protein.

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injection of MHCII targeted fusion peptides, which leads to an enhanced efficiency of peptide-presentation and a reduced binding by effector cells. Thereby the number of injections as well as the antigen dose can be reduced [11]. Besides refinement of existing forms of therapy, various newer routes for asthma treatment have been pursued. As T_H1 cells and T_H2 cells are reciprocally regulated, a multitude of studies envisaged the induction of T_H1 based immune responses in order to restrict the T_H2 responses towards allergens [12,13]. Adenoviral vector immunization has been used to induce the intra-mucosal production of the T_H1 cytokine IFN γ [14] or the upstream acting IL-18 [15] and IL-28A [16], which robustly reduced the secretion of T_H2 cytokines after stimulation with a model antigen. Furthermore, these treatments reduced asthmatic responses shortly after immunization, even after the asthmatic phenotype was fully established. Based on the results of these studies, it might be up to debate whether this effect is based only on a transient suppression of T_H2 cells, or whether there is also a longterm effect on antigen-specific T-cells. However, even a shift of the antigen-specific immune response towards T_H1 does not prevent inflammation [17] but might even lead to aggravation [18]. The notion that T_H1 polarization might be as detrimental as T_H2 polarization was supported by a recent report, showing high levels of IFN γ in severe cases of asthma, as well as in murine asthma models [19].

As major contributors to the decision between immunity and tolerance, DCs are in the focus of various immune interventions [20,21], not only regarding allergic responses. Direct targeting of antigens to surface receptors expressed by DCs, such as Mannose Receptor, DEC-205, DC-SIGN and Clec9a, leads to a specifically increased uptake, processing and presentation of the antigen by DCs [22]. Especially for DEC-205 this targeting alone does not induce complete maturation or activation of the DCs, making additional adjuvants necessary for the induction of immunity. In contrast, in the absence of adjuvants this partial maturation of DCs has led to tolerance induction, as was mainly shown in protein based approaches, for example as treatments for EAE or contact dermatitis [23–26]. The exact mechanism of this tolerizing effect has not been fully elucidated yet. However the induction of anergy in effector cells [25], induction of Tregs [26] and the induction of protective antibodies [27] have been shown in different studies. The potential of adenoviral vector based DEC-205 targeting approaches for the treatment of allergic asthma has not been elucidated yet. Nonetheless, utilizing a viral vector with the capacity for transduction of the airway mucosa provides considerable advantages in this regard.

Mucosal surfaces in general are in constant contact to foreign antigens, most of which are innocuous, thus the mucosa has to be apt for tolerance induction in order to prevent widespread immune pathology [28,29]. Furthermore a treatment locally applied to the airway mucosa would not only influence migratory cells, but would also shape the phenotype of tissue resident cells. In order to prevent anaphylactic reactions induced by a targeted antigen, dose peaks are to be avoided. However, frequent intratracheal low-dose application of a protein based treatment would be difficult, in contrast to a single dose, gene based immunization. Additionally, for practical application gene based vaccinations benefit from a greater flexibility than protein based approaches do, as the latter require extensive optimization processes for new antigens regarding production, purification and coupling to an anti-DEC-205 antibody [30].

Here, we made use of replication deficient adenoviral vectors [31], inducing the *in vivo* production of a fusion protein, consisting of OVA and a single-chain antibody against DEC-205. These were used to transduce the airway mucosa, in order to reduce the induction of an asthmatic phenotype in OVA sensitized mice before airway challenge. The targeting to DCs reduces the required antigen doses [25] and the locally confined expression might modulate more effectively the immunological environment at the mucosal surface. At the same time, the tolerogenic capacities of prolonged expression in the steady state and the DEC-targeting in the absence of immunogenic adjuvants are harnessed. Therefore the here presented work is a promising approach to finding a new treatment option for allergic asthma.

2. Material and methods

2.1. Adenoviral vectors

Replication deficient adenoviral vectors, encoding for scDEC-OVA, scGL117-OVA and GFP have been previously described [32]. In short, the respective expression cassettes were cloned into the pShuttle vector and used together with the pAdEasy system [33] to produce adenoviral particles in HEK 293A cells. Antigen expression was confirmed by western blot prior to virus purification with the Vivapure AdenoPack kit (Sartorius, Göttingen, Germany). Total particles were determined photometrically. Infectious particles were determined by the TCID₅₀ method and the typical ratio of total particles to infectious particles was approximately 100:1.

2.2. Animals

Female 6–8 week old BALB/cJ mice were purchased from Janvier (Le Genest-St-Isle, France) and housed in the central animal facility of Ruhr University Bochum at least for 14 days before experiments to allow for acclimatization. All mice were housed in accordance with the national and institutional guidelines.

2.3. Allergen induced asthma model and adenoviral immunization

On days 0 and 14 mice got intraperitoneal injections of 20 μ g OVA (grade V, Sigma, Munich, Germany) and 55 μ l Imject Alum (Pierce, Rockford, IL, USA) in a total volume of 200 μ l in sterile filtrated PBS, as a sensitization against OVA. On days 42 and 52 mice were challenged for 20 min with an aerosol of 1% OVA in sterile filtrated PBS in order to induce the asthmatic phenotype. The aerosol was generated with a PARI TurboBoy SX (PARI, Starnberg, Germany) in a Plexiglas chamber with a total volume of approximately 11 l. Immunization took place two weeks after sensitization, on day 28, by intranasal instillation of 10⁹ adenoviral particles in a total volume of 50 μ l in sterile PBS. On day 55 lung functions were measured, BALF was collected by rinsing lungs twice with 1 ml cold HBSS, and spleens and lungs were excised. The right lobes of the lungs were used for tissue sections, whereas the left lobes were used for restimulation experiments. We performed three independent experiments to demonstrate the therapeutic effect of the treatment with Ad-Dec-OVA and compared these animals to the ones from the group Asthma-Control. To further demonstrate the specific effect of the DEC205-targeting, we included in one experiment additional groups treated either with Ad-GL117-OVA or Ad-GFP. The numbers of animals per group used for the analyses are listed in Table 1.

2.4. Lung function measurements

Three days after the final challenge, mice were anesthetized with 100 mg/kg ketamine and 15 mg/kg xylazine. Spontaneous breathing was suppressed with 0.8 mg/kg Pancuronium and the airway and lung tissue reactivity to methacholine was measured using a forced oscillation technique (FlexiVent, SCIREQ, Montreal, Canada). Using the ‘quick prime 3’ (QP3) perturbation, airway resistance (Rn), tissue damping (G) and tissue elastance (H) to increasing concentrations of methacholine (0, 6.125, 12.5, 25 mg/ml) was measured. After each

Table 1
Treatment groups.

Group	Number of animals (no. of ind. experiments)
Naïve	15 (3 exp.)
Asthma-Control	16 (3 exp.)
Ad-Dec-OVA	17 (3 exp.)
Ad-GL117-OVA	6 (1 exp.)
Ad-GFP	5 (1 exp.)

concentration, the QP3 perturbation was performed 5 times spread over 2 min. If the Coefficient of Determination of a QP3 perturbation was lower than 0.90, the measurement was excluded and not used to calculate the average. For each mouse Rn, G and H were plotted against methacholine concentration and the area under the curve (AUC) was calculated to perform statistical analysis.

2.5. Analysis of BAL cells

BALs were centrifuged at 2000 ×g and the supernatant was aspirated and stored at −20 °C. Erythrocytes in the cellular fraction were lysed using ACK buffer (Lonza, Walkerville, MD, USA). Cell types were subsequently analyzed by using either the Diff-Quick kit (Labor und Technik Eberhardt Lehmann, Berlin, Germany) for differential staining, or by Flow cytometry. For FACS staining, BAL cells were incubated for 20 min at room temperature in an antibody solution containing anti-CD45-PerCP-Cy5.5, anti-CD11c-BV450, anti-CD3e-BV510, anti-CD19-PE-Cy7 (BD Bioscience), anti-CD49b-PE and anti-Gr-1-AlexaFluor488 (eBioscience). Afterwards cells were washed twice, fixed with 2% paraformaldehyde solution for 20 min at room temperature and analyzed with a FACSCanto™ II.

2.6. Antibody titers

ELISA plates were coated with 500 ng OVA in 100 µl carbonate buffer per well at 4 °C overnight. Blocking of free binding sites was performed with 5% skimmed milk in PBS containing 0.05% Tween-20 (Applichem, Darmstadt, Germany). Diluted sera or BALF samples were added and incubated for 1 h at 4 °C. As detection-antibodies, HRP coupled anti-mouse IgG1 or IgG2a, or Biotin coupled anti-mouse IgE, followed by HRP coupled Streptavidin were used (BD Bioscience, Heidelberg, Germany). Plates were developed using in house made ECL solution and luminescence was measured with a microplate luminometer (Orion L, Titertek Berthold, Pforzheim, Germany). As an internal standard, pooled sera from sensitized and challenged mice were used. The content of OVA-specific IgG1, IgG2a and IgE in this standard was defined to be 10⁷, 10⁵ and 10³ AU/ml respectively. Serial threefold dilutions of this standard were used to generate a standard curve for each ELISA plate.

2.7. OVA-specific cytokine production

Left lobes of lungs were cut into pieces of about 3 mm in diameter and treated with 250 U/ml Collagenase D (Sigma Aldrich, Munich, Germany) and 80 U/ml DNase I (Applichem) for 45 min at 37 °C. Afterwards, the lung pieces were passed through a 70 µm cell strainer in order to generate single cell suspensions. Single cell suspensions of spleens were generated by straining without previous digestion. Cells were seeded into 48-well plates at either a fixed amount of 5 × 10⁶ cells for spleens, or at a fixed volume for lungs, equivalent to one third of the total cells. Low endotoxin OVA (hyglos, Bernried, Germany) at a final concentration of 50 µg/ml was used to restimulate cells and 48 h later supernatants were taken. Ready-SET-Go! ELISA kits (eBioscience, Vienna, Austria) were used according to the manufacturer's instructions for measurement of concentrations of IL-4, IL-5, IL-10, IL-13 and IFNγ in the supernatants.

2.8. Histological sections

Three days after the final airway challenge, the right lobes of the lungs were excised and placed in cold 4% paraformaldehyde solution for fixation. The samples were embedded in paraffin-wax and tissue sections were generated using a microtome. Hematoxylin and eosin (HE) staining was used for assessment of inflammation and periodic acid schiff (PAS) staining was used to visualize mucus producing goblet cells. Stained sections were photographed at a magnification of 200- and 400-fold respectively for further analysis. Quantification of goblet

cell hyperplasia was achieved by calculation of the ratio of PAS positive cells to PAS negative cells along the basal membrane of bronchioles.

2.9. Statistical analysis

Results are shown as the mean ± standard error of the mean (SEM). For statistical comparisons GraphPad Prism 5 was used (GraphPad Software, San Diego, CA, USA) to perform one-way ANOVA or two-way ANOVA together with Tukey's post test. Results with a *p*-value ≥ 0.05 were considered not significant. * = *p* < 0.05, ** = *p* < 0.01, *** = *p* < 0.001, ns = not significant.

3. Results

3.1. Specific reduction of T_H2 cytokines without induction of T_H1 responses by DEC-targeted antigen

Sensitization of BALB/c mice by i.p. application of OVA adsorbed to alum on days 0 and 14, followed by aerosol airway challenges on days 42 and 52 (see Fig. 1A), induces a phenotype resembling the most common form of human allergen dependent asthma. This includes (but is not limited to) a T_H2 polarization of allergen-specific CD4⁺ T-cells, antigen-specific IgE production, and infiltration of the lung by eosinophilic granulocytes. Intranasal immunization with adenoviral vectors was performed on day 28, inducing the *in vivo* production of anti-mDEC-205 fused to OVA (Ad-Dec-OVA), anti-β-galactosidase fused to OVA as control antibody (Ad-GL117-OVA), or GFP as a negative control. Comparable expression levels of OVA fusion proteins as well as robust immunogenicity have been shown previously [32]. A group of sensitized and challenged, but unimmunized mice were used as positive controls for the asthmatic phenotype (Asthma-Control). Three days after the last allergen-challenge, classic T_H2-cytokines in the BAL are already reverted to or close to background levels, as was already shown by others [34,35]. Therefore, to investigate whether adenoviral vector immunization could influence the T_H2 polarization seen in Asthma-Control, unsorted single cell suspensions of cells originated from spleen or lung were restimulated for 48 h with low endotoxin OVA. Induction of an asthmatic phenotype in Asthma-Control was shown by high amounts of the classical T_H2 cytokines IL-5 and IL-13, as shown in Fig. 1B. Ad-Dec-OVA treated mice showed a significant reduction of IL-5 and IL-13 in lung, as well as in spleen, by >50% in comparison to the Asthma-Control mice. Furthermore, a T_H1-shift has not been induced, as demonstrated by only negligible levels of IFNγ. This also demonstrates that OVA-specific CD8⁺ cells are either not induced or functionally inactive. In contrast to Ad-Dec-OVA, neither Ad-GL117-OVA nor Ad-GFP reduced the amount of T_H2 cytokines produced by restimulated lung cells in comparison to Asthma-Control. Surprisingly, all three adenoviral immunizations reduced the amounts of IL-10 produced by spleen cells. Furthermore, both Ad-GL117-OVA and Ad-GFP have led to a notable amount of IFNγ, secreted by lung cells. While for Ad-GL117-OVA, this suggests a slight induction of a T_H1 response, Ad-GFP also increased the amounts of IL-4 in the lung, indicating a generally pro-inflammatory effect. This is in line with a study using a similar adenovirus coding for GFP [36].

3.2. DEC-targeting of antigen reduces lung eosinophilia

IL-5 and IL-13 are critical for the recruitment of eosinophils and their persistence [37,38], therefore we analyzed the effect of our adenoviral immunization strategies on the cellular composition in the BAL by flow cytometry, shown in Fig. 2B. In naïve controls, alveolar macrophages were by far the predominant cell type, comprising 72.3% of all cells. In contrast, in Asthma-Control infiltration of mainly eosinophilic granulocytes, as well as a substantial amount of lymphocytes, reduced the percentage of macrophages to <5% in the BAL. Due to this infiltration, eosinophils amounted to 70.1% of BAL cells in Asthma-Control,

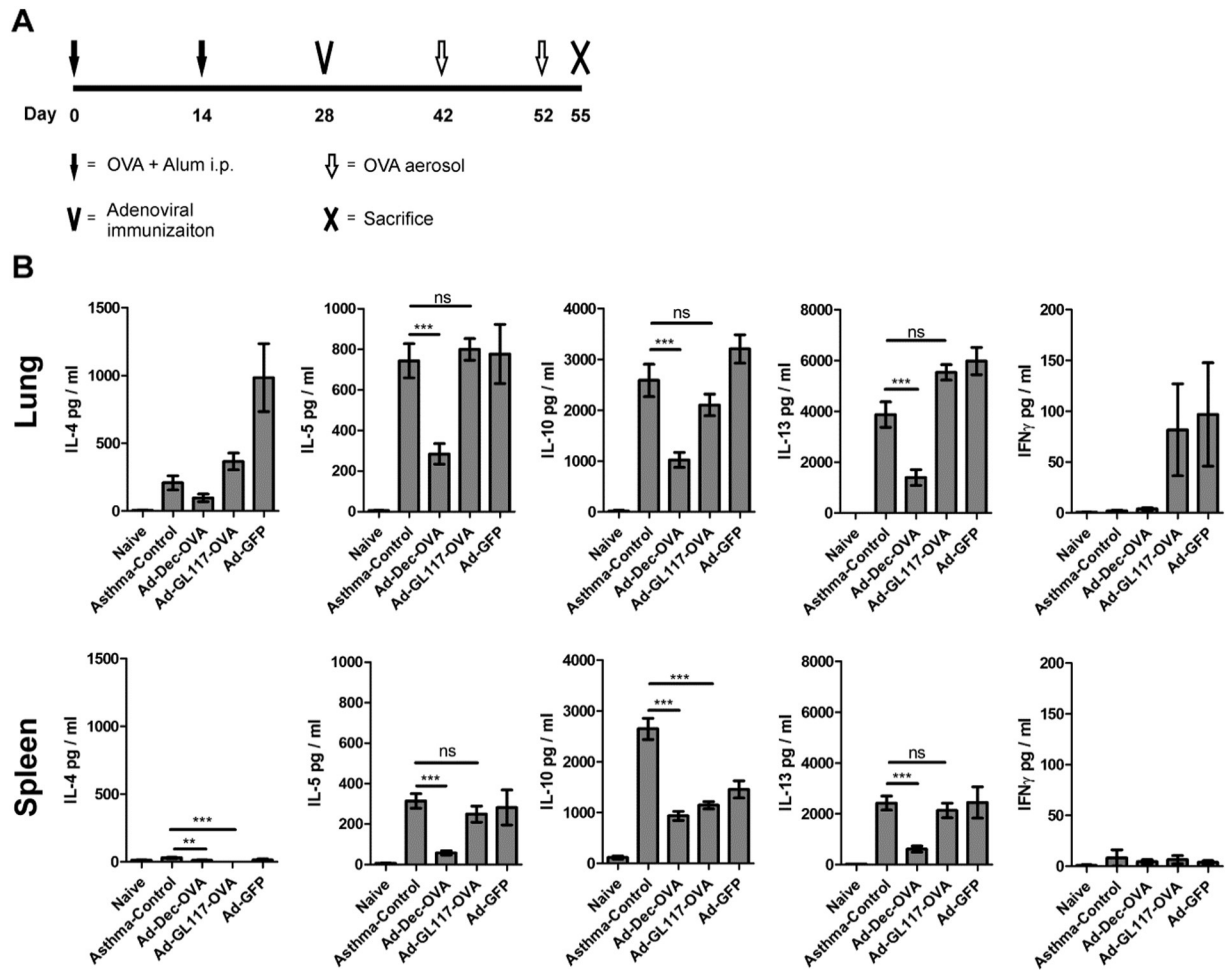


Fig. 1. A Timeline for the induction of the asthmatic phenotype and the adenoviral vector immunization. B Single cell suspensions from lungs or spleens were stimulated with OVA protein for 48 h and the amounts of indicated cytokines in the supernatant were determined by colorimetric ELISA. Each bar represents the mean concentration and the respective SEM of 5–17 mice according to Table 1. Since IFN- γ was hardly detectable at all; the IFN- γ ELISA was only performed once and represent 5–6 animals per group. ** = $p < 0.01$, *** = $p < 0.001$, ns = not significant.

while 20.1% of the detected cells were lymphocytes. In comparison, in Ad-Dec-OVA treated mice, eosinophils were reduced to 38.7%, resulting in a significant increase in the percentage of both lymphocytes and macrophages (38.5% and 15.1% respectively). However, this increase in the percentage of lymphocytes and macrophages is not accompanied by an increase in the total numbers. In fact, the total number of lymphocytes is not significantly different in Ad-Dec-OVA treated mice in

comparison with Asthma-Control, but the total number of eosinophils is reduced by 75% due to the immunization with Ad-Dec-OVA (see Fig. S1). In line with these numbers, we also observed lower levels of eotaxin-1, one of the eosinophil chemotactic proteins, in the BAL of Ad-Dec-OVA treated animals (see Fig. S2). In contrast, immunization with adenoviral vectors inducing the production of GL117-OVA or GFP did not lead to significant changes in the cellular composition in

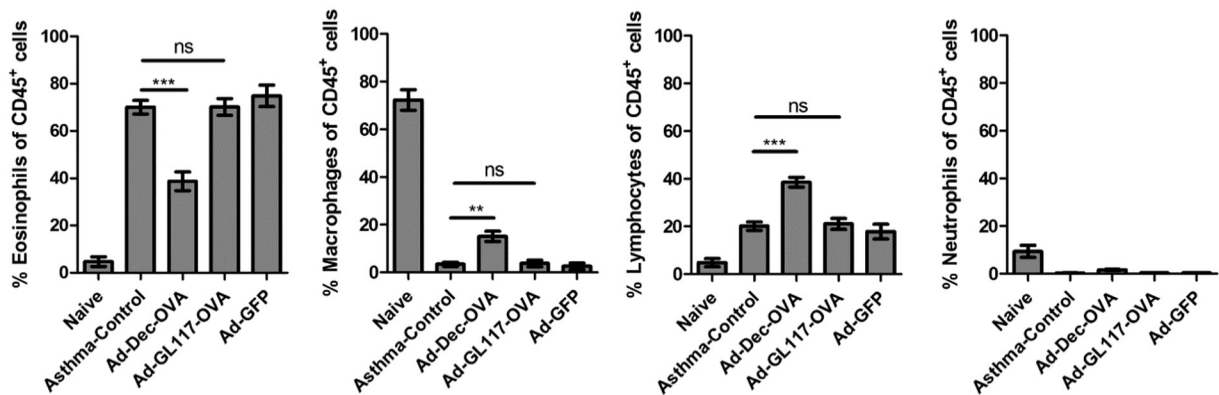


Fig. 2. FACS analysis of cells in BAL. A BAL was performed three days after the last airway challenge and the cellular content was analyzed by FACS for the indicated cell types. Each bar represents the mean percentage among CD45 $^{+}$ cells and the respective SEM of 5–17 mice according to Table 1. Due to technical problems, three samples of the group Naive and two of the group Asthma-Control could not be properly processed, leaving $n = 12$ and $n = 14$ for these groups. ** = $p < 0.01$, *** = $p < 0.001$, ns = not significant.

comparison to Asthma-Control. Importantly, the reduced eosinophilia upon Ad-Dec-OVA treatment did not lead to the induction of airway neutrophilia.

3.3. Reduced antigen-specific IgE in Ad-Dec-OVA immunized mice

Allergies and asthma are closely related to increased titers in IgE, especially in the involved tissues [39], which prompted us to analyze the content of anti-OVA IgE in the BALF by ELISA, shown in Fig. 3. In contrast to naïve mice, in which IgE was below the detection limit, mice from the group Asthma-Control showed robust amounts of OVA-specific IgE in BALF (for definition of the arbitrary units, see method section). In comparison, the IgE titers in Ad-Dec-OVA treated animals were reduced by 85%, whereas Ad-GL117-OVA and Ad-GFP treated mice showed IgE levels comparable to the group Asthma-Control. Furthermore, we analyzed the titers of OVA-specific IgG1 and IgG2a as an additional assessment for a possible T_H1 shift, but no significant differences between OVA sensitized groups were observed. In sera, no effect of adenoviral immunization on OVA specific IgG1, IgG2a or IgE was detectable (data not shown).

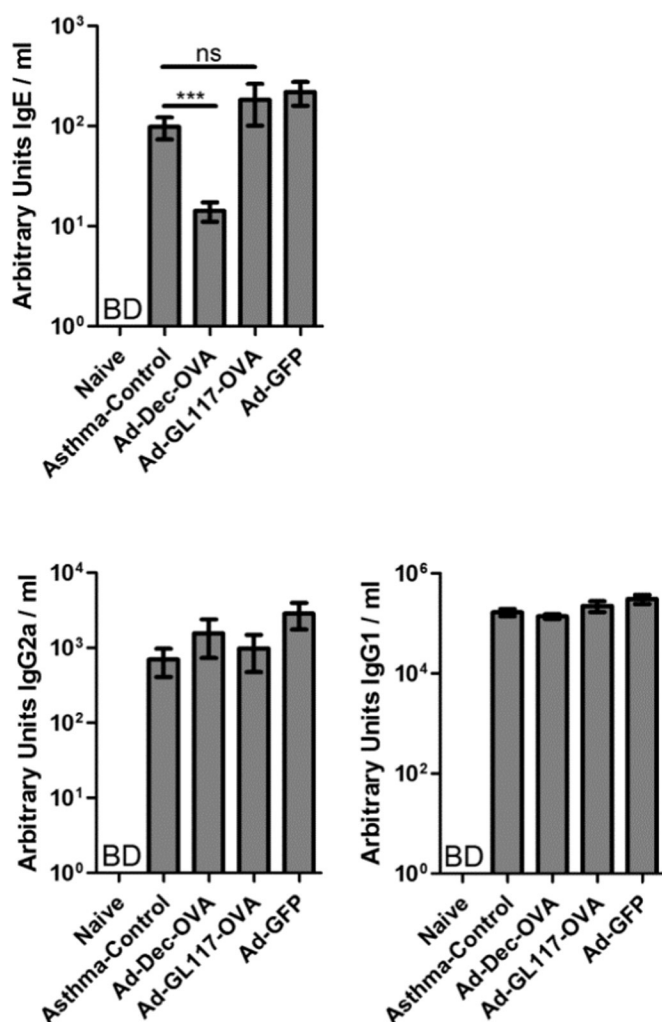


Fig. 3. OVA-specific antibodies in BALF. BALs were performed three days after the final challenge and OVA-specific antibodies were measured by ELISA. The amount of IgE (A), IgG2a (B) and IgG1 (C) is shown. BD = below detection limit. Each bar represents mean data and SEM from 5 to 17 mice according to Table 1. *** = $p < 0.001$, ns = not significant.

3.4. Reduced goblet cell hyperplasia after Ad-Dec-OVA immunization

A common symptom of asthmatic patients is a marked hyperplasia and activation of mucus producing goblet cells, which correlates with mortality [40]. Therefore, we analyzed whether the immunological ameliorations described above would translate into a decrease in goblet cell numbers. Three days after the last airway challenge, the right lobes of the lungs were excised, fixated in paraformaldehyde and embedded in paraffin. Tissue sections were generated and PAS-stained. In Fig. 4A, representative micrographs are shown. In naïve mice, PAS positive cells, representing mucus producing goblet cells, were completely absent, whereas in mice from the group Asthma-Control, a striking goblet cell hyperplasia was seen. In contrast, in Ad-Dec-OVA immunized mice, only minute amounts of PAS positive cells were observable. Ad-GL117-OVA treated mice also showed a reduction of goblet cell hyperplasia in comparison to Asthma-Control, but not as pronounced as seen in the Ad-Dec-OVA group. Lastly, in Ad-GFP treated mice a change in goblet cell hyperplasia was hardly noticeable. In order to quantify the reduction in PAS positive cells, we calculated the ratio of goblet cells to epithelial cells along the basal membrane, shown in Fig. 4B. The group Asthma-Control showed a mean goblet to epithelial cell ratio of 0.90, whereas Ad-Dec-OVA mice had a mean of 0.08. Quantification of mRNA for Gob5, a mucus-related gene, from these sections by qRT-PCR also revealed higher levels for the group Asthma-Control and the Ad-GL117-OVA in comparison to the mRNA levels in the lung of Ad-Dec-OVA treated animals (data not shown).

3.5. Ad-Dec-OVA immunization improves lung function

After establishing the ameliorating effect of Ad-Dec-OVA on immunological and histological parameters of the asthma like phenotype, we wanted to elucidate whether this translates into an improved lung function. Anesthetized mice were tracheotomized and lung function was assessed by forced oscillation technique with inhalation of escalating doses of the bronchoconstrictor methacholine as an aerosol. In Fig. 5A–C, the increase of airway resistance (R_N), tissue damping (G) and tissue elastance (H) in response to methacholine is shown. All four included groups (Naïve, Asthma-Control, Ad-Dec-OVA and Ad-GL117-OVA) showed a similarly modest increase in R_N . The areas under the curve (AUC) for R_N of the sensitized groups did not differ significantly from the Naïve group. In contrast, for both tissue damping and elastance, mice from the group Asthma-Control and Ad-GL117-OVA treated mice showed a significantly higher increase than Ad-Dec-OVA mice did (Fig. 5D–F), which was already observable at low doses of methacholine. Thus, an overall improved lung function in Ad-Dec-OVA treated mice, but not mice treated with Ad-GL117-OVA was indeed shown.

4. Discussion

Adenoviral vectors have proven to be efficient for transient immune manipulations. Their considerable capacity for the transduction of the airway mucosa established them as a potent tool for the local induction of T_H1 polarizing agents. Although these approaches were highly efficacious as short term treatments in allergic asthma models [14–16], they lack intrinsic allergen specificity. Therefore, long term effects would either be expected to be absent or would have to stem from unspecific reprogramming of the local immune system. As a consequence, T_H1 polarizing treatments would pose the risk of uncontrolled T_H1 -based immune reactions towards the allergen [17–19], as well as other innocuous antigens. In contrast, SIT aims for the induction of specific tolerance, but has a noticeably long treatment duration. In the present study, we utilized adenoviral vectors in order to induce allergen-specific tolerance *in situ* by secretion of a fusion protein, consisting of the allergen and a single-chain antibody against DEC-205. Targeting towards DCs via DEC-205 not only reduces the required amount of allergen [41], which is highly beneficial for the safety profile, but also acts

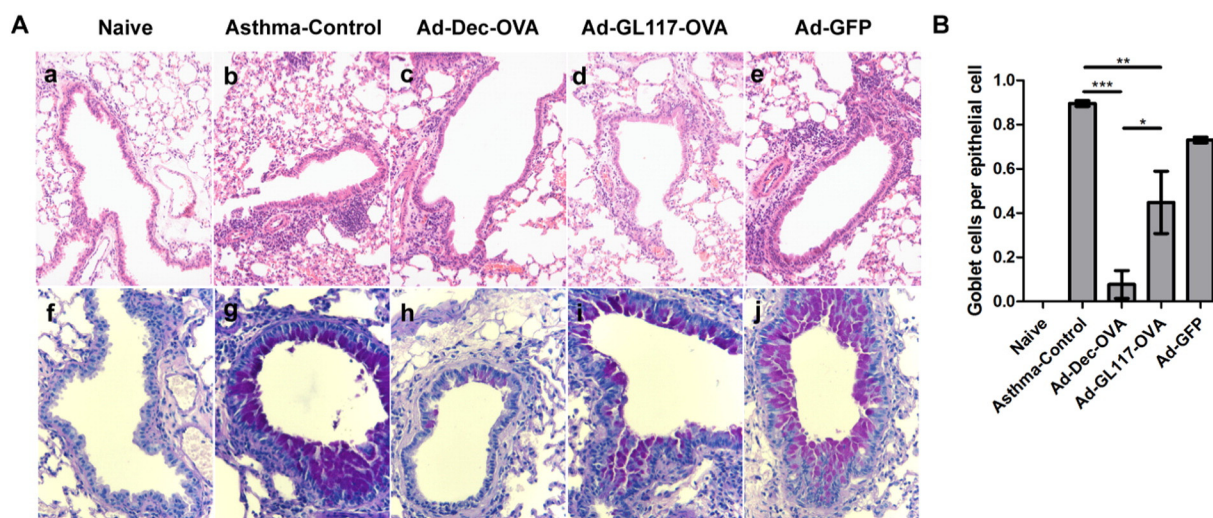


Fig. 4. Histological examination. Three days after the last airway challenge, the right halves of the lungs were excised and fixated in paraformaldehyde. After fixation, lungs were embedded in paraffin wax and sliced using a microtome. Subsequently, slices were stained for histological examination. Magnification for hematoxylin and eosin (HE) stained samples 200 fold and for periodic acid Schiff (PAS) 400 fold. A HE (a-e) and PAS (f-j) staining. From left to right: Naive, Asthma-Control, Ad-Dec-OVA, Ad-GL117-OVA and Ad-GFP. B Ratio of PAS⁺ goblet cells to epithelial cells along the basal membrane. Each bar represents the mean of five sections from each of three mice per group with the respective SEM. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, ns = not significant.

tolerogenic in the absence of adjuvants [25]. Protein based adaptations of this strategy have been successfully used as treatments in murine models of diabetes [24], contact dermatitis [26] and multiple sclerosis [42]. Furthermore, in another recent study, a DNA based vaccine, encoding for a human collagen-domain coupled to anti-DEC-205, reduced rejection of human collagen-transgenic skin grafts in mice [43]. We have previously shown that a DNA based systemic immunization encoding for allergens targeted to DEC-205 has a tolerizing effect. However, lung resident cells were not sufficiently affected in order to treat an asthmatic phenotype [44] which highlights the importance of the elicitation of local immune modifications in the lung mucosa for the treatment of allergic asthma. Indeed, in the present study, we found the T_H2 inducing cytokine IL-4 to be produced in considerable amounts only by cells from the lung, but not from the spleen. The same was true for the production of IFN γ in mice immunized with Ad-GL117-OVA or Ad-GFP, which are the only groups that showed notable IFN γ production at all. This absence of IFN γ production in Ad-Dec-OVA immunized mice demonstrates the avoidance of T_H1 mediated inflammation, which is further supported by the absence of increased OVA-specific IgG2a titers. At the same time the risk of type I hypersensitivity reactions, and thus anaphylaxis, is considerably low, taking into account that OVA-specific lung IgE titers are reduced by 85% in Ad-Dec-OVA immunized mice. Again, this reduction was only observed in the lung, supporting the hypothesis that local immune modulations are highly important. It has been shown previously that IgE is produced locally after allergen challenge in mouse models [45], as well as in human patients [46]. Nevertheless, said possible tolerance induction is exemplified by the reduction of the T_H2 cytokines IL-5 and IL-13 concurrent to the reduction of IFN γ , in comparison to Ad-GL117-OVA and Ad-GFP. At least partially as a result of the decrease in IL-5 and IL-13, eosinophilic granulocytes are reduced in the BAL of Ad-Dec-OVA treated animals, as IL-5 and IL-13 are crucial for the induction and survival of eosinophils [37,38,47]. These reduced eosinophile numbers, together with the decreased OVA-specific IgE titers, will consequently lessen subsequent eosinophilic degranulation and thus total inflammation in response to inhalation of the allergen. In accordance with these findings, goblet cell hyperplasia and AHR are also reduced in Ad-Dec-OVA treated mice. The here obtained results for the lung parameters R_N, G and H are in line with a study that used a mouse model that is highly comparable to our Naive and Asthma-Control groups, in order to compare forced oscillation technique and alveolar capsules for assessment of

AHR [48]. Furthermore, Riesenfeld et al. noted a time dependent component regarding the site of inflammation, with increases in R_N early after allergen challenge and a shift towards predominantly increases in G and H at 48 h after the last allergen challenge [49]. Interesting in this regard is the occurrence of only minute increases in the resistance of the conducting airways R_N in mice from the group Asthma-Control, despite the presence of bronchioles that are highly occluded by PAS⁺ goblet cells in our study. This has general implications for the induced asthmatic phenotype and indicates a predominantly peripheral and heterogeneous inflammation, as the effect of occluded distal airways on the total airway resistance is diminished if parallel conducting airways are unaffected [50]. Furthermore, increases in lung elastance H and tissue damping G are in general associated with peripheral inflammation [51]. Tissue distortions or the occurrence of edema for example, can increase H in a widespread area, where compensations as suggested for R_N would not be as easily possible. Moreover, with a uniformly distributed elastance and heterogeneous resistance of the corresponding conducting airways, so called “pendelluft” occurs, which signifies the movement of air back and forth between lung compartments. This leads to respiratory efforts that do not result in an increased net airflow at the airway opening and would therefore explain at least parts of the increase in the energy dissipative parameter G, as seen in this study [52]. Based on these considerations, we reason that the asthmatic phenotype induced by our protocol is characterized by a homogeneous effect on the tissue compartment and a constriction of the conducting airways that is heterogeneous as well as restricted to the periphery.

In conclusion, we showed as a proof of principle that mucosally delivered viral vectors encoding antigens, fused to a single chain antibody against DEC-205, are a feasible means to alleviate the symptoms of an allergic asthma model in an antigen-specific manner, even after established sensitization. A single dose vaccination exerted a significant beneficial effect in regard to immunological, histological and lung function parameters that was not seen with untargeted antigen. Furthermore, we showed that this vaccination scheme reduced the T_H2 polarization without the induction of a potentially deleterious T_H1 response. Together with the observed reduction in allergen-specific IgE and in eosinophilic granulocytes, we consider this vaccination to be sufficiently safe, although the underlying mechanism should be addressed in further studies. In regard to the pre-existing immunity to AdV in the human population, the use of rare serotypes of adenoviruses instead of AdV might avoid decreased efficacy due to neutralization of the vaccine

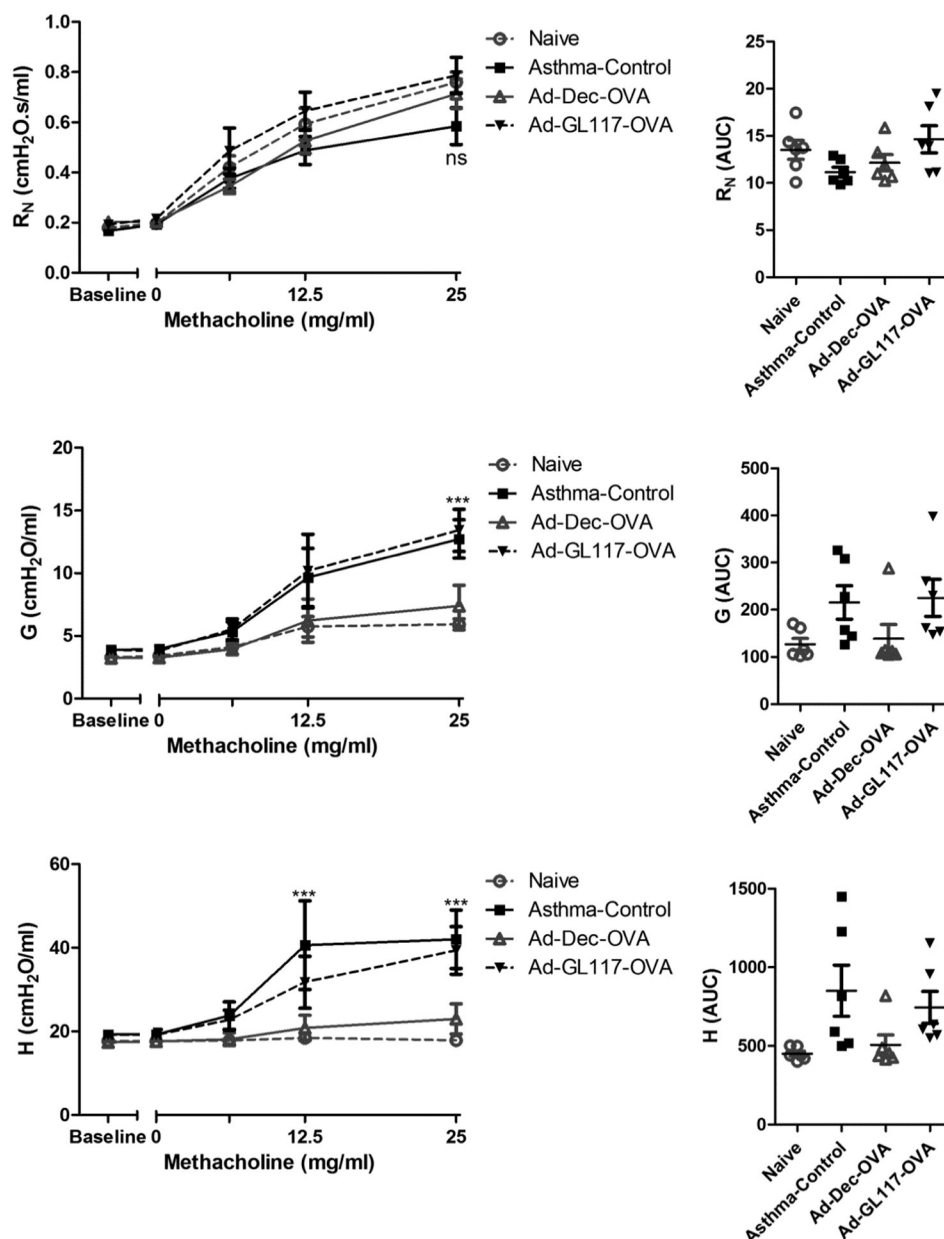


Fig. 5. Effect of immunization with Ad-Dec-OVA on lung function. Three days after the last airway challenge, deeply anesthetized mice were tracheotomized and connected to a Flexivent small animal ventilator. Forced oscillation maneuvers were performed with or without prior inhalation of methacholine solutions with escalating concentrations. A–C influence of different doses of methacholine on airway resistance R_N (A), tissue damping G (B) and tissue elastance H (C) D–F corresponding areas under the curve. Each bar represents the mean of six mice per group with the respective SEM. * = $p < 0.05$, ** = $p < 0.01$, ns = not significant.

vector. Additionally, other viral vectors which results in constant antigen levels after mucosal delivery, like adeno-associated viruses, might represent an interesting alternative to analyze in future studies.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jconrel.2016.06.043>.

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References

- [1] B.N. Lambrecht, H. Hammad, The immunology of asthma, *Nat. Immunol.* 16 (2014) 45–56, <http://dx.doi.org/10.1038/ni.3049>.
- [2] L. Maddox, D.A. Schwartz, The pathophysiology of asthma, *Annu. Rev. Med.* 53 (2002) 477–498, <http://dx.doi.org/10.1146/annurev.med.53.082901.103921>.
- [3] P.J. Barnes, Intrinsic asthma: not so different from allergic asthma but driven by superantigens? *Clin. Exp. Allergy* 39 (2009) 1145–1151, <http://dx.doi.org/10.1111/j.1365-2222.2009.03298.x>.
- [4] P.G. Woodruff, B. Modrek, D.F. Choy, G. Jia, A.R. Abbas, A. Ellwanger, L.L. Koth, J.R. Arron, J.V. Fahy, T-helper type 2-driven inflammation defines major subphenotypes of asthma, *Am. J. Respir. Crit. Care Med.* 180 (2009) 388–395, <http://dx.doi.org/10.1164/rccm.200903-0392OC>.
- [5] B. Bosnjak, B. Stelzmueller, K.J. Erb, M.M. Epstein, Treatment of allergic asthma: modulation of Th2 cells and their responses, *Respir. Res.* 12 (2011) 114, <http://dx.doi.org/10.1186/1465-9921-12-114>.
- [6] A.J. Frew, Allergen immunotherapy, *J. Allergy Clin. Immunol.* 125 (2010) S306–S313, <http://dx.doi.org/10.1016/j.jaci.2009.10.064>.

- [7] G.W. Canonica, D. Bagnasco, G. Ferrantino, M. Ferrando, G. Passalacqua, Update on immunotherapy for the treatment of asthma, *Curr. Opin. Pulm. Med.* 22 (2016) 18–24, <http://dx.doi.org/10.1097/MCP.0000000000000227>.
- [8] J. Dretzke, A. Meadows, N. Novielli, A. Huisssoon, A. Fry-Smith, C. Meads, Subcutaneous and sublingual immunotherapy for seasonal allergic rhinitis: a systematic review and indirect comparison, *J. Allergy Clin. Immunol.* 131 (2013) 1361–1366, <http://dx.doi.org/10.1016/j.jaci.2013.02.013>.
- [9] N. Mothes-Luksch, S. Stumvoll, B. Linhart, M. Focke, M.-T. Krauth, A. Hauswirth, P. Valent, P. Verdino, T. Pavkov, W. Keller, M. Grote, R. Valenta, Disruption of allergenic activity of the major grass pollen allergen Phl p 2 by reassembly as a mosaic protein, *J. Immunol.* 181 (2008) 4864–4873 (<http://www.ncbi.nlm.nih.gov/pubmed/18802090>) (accessed September 2, 2015).
- [10] M. Curin, M. Weber, T. Thalhamer, I. Swoboda, M. Focke-Tejkl, K. Blatt, P. Valent, K. Marth, T. Garmatiuk, H. Grönlund, J. Thalhamer, S. Spitzauer, R. Valenta, Hypoallergenic derivatives of Fel d 1 obtained by rational reassembly for allergy vaccination and tolerance induction, *Clin. Exp. Allergy* 44 (2014) 882–894, <http://dx.doi.org/10.1111/cea.12294>.
- [11] A. Zaleska, T. Eiwegger, O. Soyer, W. van de Veen, C. Rhyner, M.B. Soyka, C. Bekpen, D. Demiröz, A. Treis, S. Söllner, O. Palomares, W.W. Kwok, H. Rose, G. Senti, T.M. Kündig, N. Ozoren, M. Jutel, C.A. Akdis, R. Cramer, M. Akdis, Immune regulation by intralymphatic immunotherapy with modular allergen translocation MAT vaccine, *Allergy* 69 (2014) 1162–1170, <http://dx.doi.org/10.1111/all.12461>.
- [12] C.C. Wang, G.A. Rook, Inhibition of an established allergic response to ovalbumin in BALB/c mice by killed mycobacterium vaccae, *Immunology* 93 (1998) 307–313 (<http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1364077&tool=pmcentrez&rendertype=abstract>) (accessed September 4, 2015).
- [13] M.R. Stämpfli, S.A. Ritz, G.S. Neigh, P.J. Sime, X.F. Lei, Z. Xing, K. Croitoro, M. Jordana, Adenoviral infection inhibits allergic airways inflammation in mice, *Clin. Exp. Allergy* 28 (1998) 1581–1590 (<http://www.ncbi.nlm.nih.gov/pubmed/10024231>) (accessed September 4, 2015).
- [14] A.K. Behera, M. Kumar, R.F. Lockey, S.S. Mohapatra, Adenovirus-mediated interferon gamma gene therapy for allergic asthma: involvement of interleukin 12 and STAT4 signaling, *Hum. Gene Ther.* 13 (2002) 1697–1709, <http://dx.doi.org/10.1089/104303402760293547>.
- [15] D.M. Walter, C.P. Wong, R.H. DeKruyff, G.J. Berry, S. Levy, D.T. Umetsu, IL-18 gene transfer by adenovirus prevents the development of and reverses established allergen-induced airway hyperreactivity, *J. Immunol.* 166 (2001) 6392–6398 (<http://www.ncbi.nlm.nih.gov/pubmed/11342664>) (accessed September 3, 2015).
- [16] O. Koltsida, M. Hausding, A. Stavropoulos, S. Koch, G. Tzelepis, C. Ubel, S.V. Kottenko, P. Sideras, H.A. Lehr, M. Tepe, K.M. Klucher, S.E. Doyle, M.F. Neurath, S. Finotto, E. Andreaskos, IL-28 A (IFN- λ 2) modulates lung DC function to promote Th1 immune skewing and suppress allergic airway disease, *EMBO Mol. Med.* 3 (2011) 348–361, <http://dx.doi.org/10.1002/emmm.201100142>.
- [17] D.A. Randolph, C.J. Carruthers, S.J. Szabo, K.M. Murphy, D.D. Chaplin, Modulation of airway inflammation by passive transfer of allergen-specific Th1 and Th2 cells in a mouse model of asthma, *J. Immunol.* 162 (1999) 2375–2383 (<http://www.ncbi.nlm.nih.gov/pubmed/9973518>) (accessed September 3, 2015).
- [18] G. Hansen, G. Berry, R.H. DeKruyff, D.T. Umetsu, Allergen-specific Th1 cells fail to counterbalance Th2 cell-induced airway hyperactivity but cause severe airway inflammation, *J. Clin. Invest.* 103 (1999) 175–183, <http://dx.doi.org/10.1172/JCI5155>.
- [19] M. Raundhal, C. Morse, A. Khare, T.B. Oriss, J. Milosevic, J. Trudeau, R. Huff, J. Pilewski, F. Holguin, J. Kolls, S. Wenzel, P. Ray, A. Ray, High IFN- γ and low SLP1 mark severe asthma in mice and humans, *J. Clin. Invest.* 125 (2015) 3037–3050, <http://dx.doi.org/10.1172/JCI80911>.
- [20] K. Palucka, J. Banchereau, Cancer immunotherapy via dendritic cells, *Nat. Rev. Cancer* 12 (2012) 265–277, <http://dx.doi.org/10.1038/nrc3258>.
- [21] C.C. Gross, H. Wiendl, Dendritic cell vaccination in autoimmune disease, *Curr. Opin. Rheumatol.* 25 (2013) 268–274, <http://dx.doi.org/10.1097/BOR.0b013e32835cb9f2>.
- [22] M. Kreutz, P.J. Tacke, C.G. Figdor, Targeting dendritic cells—why bother? *Blood* 121 (2013) 2836–2844, <http://dx.doi.org/10.1182/blood-2012-09-452078>.
- [23] J.N.H. Stern, D.B. Keskin, Z. Kato, H. Waldner, S. Schallenberg, A. Anderson, H. von Boehmer, K. Kretschmer, J.L. Strominger, Promoting tolerance to proteolipid protein-induced experimental autoimmune encephalomyelitis through targeting dendritic cells, *Proc. Natl. Acad. Sci. U. S. A.* 107 (2010) 17280–17285, <http://dx.doi.org/10.1073/pnas.1010263107>.
- [24] D. Bruder, A.M. Westendorf, W. Hansen, S. Pretti, A.D. Gruber, Y. Qian, H. von Boehmer, K. Mahnke, J. Buer, On the edge of autoimmunity: T-cell stimulation by steady-state dendritic cells prevents autoimmune diabetes, *Diabetes* 54 (2005) 3395–3401 (<http://www.ncbi.nlm.nih.gov/pubmed/16306354>) (accessed September 3, 2015).
- [25] D. Hawiger, K. Inaba, Y. Dorsett, M. Guo, K. Mahnke, M. Rivera, J.V. Ravetch, R.M. Steinman, M.C. Nussenzweig, Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo, *J. Exp. Med.* 194 (2001) 769–779 (<http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2195961&tool=pmcentrez&rendertype=abstract>) (accessed June 29, 2015).
- [26] K. Mahnke, Y. Qian, J. Knop, A.H. Enk, Induction of CD4 + CD25 + regulatory T cells by targeting of antigens to immature dendritic cells, *Blood* 101 (2003) 4862–4869, <http://dx.doi.org/10.1182/blood-2002-10-3229>.
- [27] C.M. Oefner, A. Winkler, C. Hess, A.K. Lorenz, V. Holeccka, M. Huxdorf, T. Schommartz, D. Petzold, J. Bitterling, A.-L. Schoen, A.D. Stoehr, D.V. Van, Y. Darcan-Nikolaïsen, V. Blanchard, I. Schmudde, Y. Laumonier, H.A. Ströver, A.N. Hegazy, S. Eiglmeier, C.T. Schoen, M.M.M. Mertes, C. Lodenkemper, M. Löhning, P. König, A. Petersen, E.O. Luger, M. Collin, J. Köhl, A. Hutloff, E. Hamelmann, M. Berger, H. Wardemann, M. Ehlers, Tolerance induction with T cell-dependent protein antigens induces regulatory sialylated IgGs, *J. Allergy Clin. Immunol.* 129 (6) (2012) 1647–1655, <http://dx.doi.org/10.1016/j.jaci.2012.02.037>.
- [28] P.G. Holt, C. McMenamin, Defence against allergic sensitization in the healthy lung: the role of inhalation tolerance, *Clin. Exp. Allergy* 19 (1989) 255–262 (<http://www.ncbi.nlm.nih.gov/pubmed/2660967>) (accessed July 2, 2015).
- [29] T.B. Tomasi, Mechanisms of immune regulation at mucosal surfaces, *Rev. Infect. Dis.* 5 (Suppl. 4) (1983) S784–2, <http://www.ncbi.nlm.nih.gov/pubmed/6227073> (accessed July 2, 2015).
- [30] C. Grossmann, M. Tenbusch, G. Nchinda, V. Temchura, G. Nabi, G.W. Stone, R.S. Kornbluth, K. Überla, Enhancement of the priming efficacy of DNA vaccines encoding dendritic cell-targeted antigens by synergistic toll-like receptor ligands, *BMC Immunol.* 10 (2009) 43, <http://dx.doi.org/10.1186/1471-2172-10-43>.
- [31] J. Luo, Z.-L. Deng, X. Luo, N. Tang, W.-X. Song, J. Chen, K.A. Sharff, H.H. Luu, R.C. Haydon, K.W. Kinzler, B. Vogelstein, T.-C. He, A protocol for rapid generation of recombinant adenoviruses using the AdEasy system, *Nat. Protoc.* 2 (2007) 1236–1247, <http://dx.doi.org/10.1038/nprot.2007.135>.
- [32] M. Tenbusch, G. Nchinda, M. Storcksdieck genannt Bonsmann, V. Temchura, K. Überla, Targeting the antigen encoded by adenoviral vectors to the DEC205 receptor modulates the cellular and humoral immune response, *Int. Immunol.* 25 (2013) 247–258, <http://dx.doi.org/10.1093/intimm/dxs112>.
- [33] T.C. He, S. Zhou, L.T. da Costa, J. Yu, K.W. Kinzler, B. Vogelstein, A simplified system for generating recombinant adenoviruses, *Proc. Natl. Acad. Sci. U. S. A.* 95 (1998) 2509–2514 (<http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=193948&tool=pmcentrez&rendertype=abstract>) (accessed July 8, 2015).
- [34] I. Meyts, P.W. Hellings, G. Hens, B.M. Vanaudenaerde, B. Verbinen, H. Heremans, P. Matthys, D.M. Bullens, L. Overbergh, C. Mathieu, K. De Boeck, J.L. Ceuppens, IL-12 contributes to allergen-induced airway inflammation in experimental asthma, *J. Immunol.* 177 (2006) 6460–6470, <http://dx.doi.org/10.4049/jimmunol.177.9.6460>.
- [35] J. Kim, L. McKinley, S. Natarajan, G.L. Bolgos, J. Siddiqui, S. Copeland, D.G. Remick, Anti-tumor necrosis factor- α antibody treatment reduces pulmonary inflammation and methacholine hyper-responsiveness in a murine asthma model induced by house dust, *Clin. Exp. Allergy* 36 (2006) 122–132, <http://dx.doi.org/10.1111/j.1365-2222.2005.02407.x>.
- [36] F. Re, R. Srinivasan, T. Igarashi, F. Marincola, R. Childs, Green fluorescent protein expression in dendritic cells enhances their immunogenicity and elicits specific cytotoxic T-cell responses in humans, *Exp. Hematol.* 32 (2004) 210–217, <http://dx.doi.org/10.1016/j.jexphem.2003.10.014>.
- [37] S. Horie, Y. Okubo, M. Hossain, E. Sato, H. Nomura, S. Koyama, J. Suzuki, M. Isobe, M. Sekiguchi, Interleukin-13 but not interleukin-4 prolongs eosinophil survival and induces eosinophil chemotaxis, *Intern. Med.* 36 (1997) 179–185 (<http://www.ncbi.nlm.nih.gov/pubmed/9144009>) (accessed July 9, 2015).
- [38] P.D. Collins, S. Marleau, D.A. Griffiths-Johnson, P.J. Jose, T.J. Williams, Cooperation between interleukin-5 and the chemokine eotaxin to induce eosinophil accumulation in vivo, *J. Exp. Med.* 182 (1995) 1169–1174 (<http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2192289&tool=pmcentrez&rendertype=abstract>) (accessed June 12, 2015).
- [39] H.J. Gould, B.J. Sutton, IgE in allergy and asthma today, *Nat. Rev. Immunol.* 8 (2008) 205–217, <http://dx.doi.org/10.1038/nri2273>.
- [40] T. Aikawa, S. Shimura, H. Sasaki, M. Ebina, T. Takishima, Marked goblet cell hyperplasia with mucus accumulation in the airways of patients who died of severe acute asthma attack, *Chest* 101 (1992) 916–921 (<http://www.ncbi.nlm.nih.gov/pubmed/1555462>) (accessed July 6, 2015).
- [41] L. Bonifaz, D. Bonnyay, K. Mahnke, M. Rivera, M.C. Nussenzweig, R.M. Steinman, Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8 + T cell tolerance, *J. Exp. Med.* 196 (2002) 1627–1638 (<http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2196060&tool=pmcentrez&rendertype=abstract>) (accessed October 16, 2015).
- [42] S. Ring, M. Maas, D.M. Nettelbeck, A.H. Enk, K. Mahnke, Targeting of autoantigens to DEC205 + dendritic cells in vivo suppresses experimental allergic encephalomyelitis in mice, *J. Immunol.* 191 (2013) 2938–2947, <http://dx.doi.org/10.4049/jimmunol.1202592>.
- [43] M. Ettinger, I.K. Gratz, C. Gruber, C. Hauser-Kronberger, T.S. Johnson, K. Mahnke, J. Thalhamer, H. Hintner, D. Peckl-Schmid, J.W. Bauer, Targeting of the hNC16A collagen domain to dendritic cells induces tolerance to human type XVII collagen, *Exp. Dermatol.* 21 (2012) 395–398, <http://dx.doi.org/10.1111/j.1600-0625.2012.01474.x>.
- [44] T. Niezold, M. Storcksdieck Genannt Bonsmann, A. Maaske, V. Temchura, V. Heinecke, D. Hannaman, J. Buer, C. Ehrhardt, W. Hansen, K. Überla, M. Tenbusch, DNA vaccines encoding DEC205-targeted antigens: immunity or tolerance? *Immunology* 145 (2015) 519–533, <http://dx.doi.org/10.1111/imm.12467>.
- [45] Y. Chvatchko, M.H. Kosco-Vilbois, S. Herren, J. Lefort, J.Y. Bonnefoy, Germinal center formation and local immunoglobulin E (IgE) production in the lung after an airway antigenic challenge, *J. Exp. Med.* 184 (1996) 2353–2360 (<http://www.ncbi.nlm.nih.gov/pubmed/8976189>) (accessed June 28, 2016).
- [46] L. Smurthwaite, S.R. Durham, Local IgE synthesis in allergic rhinitis and asthma, *Curr. Allergy Asthma Rep.* 2 (2002) 231–238 (<http://www.ncbi.nlm.nih.gov/pubmed/11918865>) (accessed June 28, 2016).
- [47] M. Yang, S.P. Hogan, S. Mahalingam, S.M. Pope, N. Zimmermann, P. Fulkerson, L.A. Dent, I.G. Young, K.I. Mattheai, M.E. Rothenberg, P.S. Foster, Eotaxin-2 and IL-5 cooperate in the lung to regulate IL-13 production and airway eosinophilia and hyperactivity, *J. Allergy Clin. Immunol.* 112 (2003) 935–943, <http://dx.doi.org/10.1016/j.jaci.2003.08.010>.
- [48] S. Tomioka, J.H.T. Bates, C.G. Irvin, Airway and tissue mechanics in a murine model of asthma: alveolar capsule vs. forced oscillations, *J. Appl. Physiol.* 93 (2002) 263–270, <http://dx.doi.org/10.1152/japplphysiol.01129.2001>.
- [49] E. Riesenfeld, G.B. Allen, J.H. Bates, M.E. Poynter, M. Wu, S. Aimiand, L.K. Lundblad, The temporal evolution of airways Hyperresponsiveness and inflammation, *J. Allergy Ther.* 1 (2012) 1–7, <http://dx.doi.org/10.4172/2155-6121.S1-005>.

- [50] A.B. Otis, C.B. Mckerrow, R.A. Bartlett, J. Mead, M.B. McIlroy, N.J. Selver-Stone, E.P. Radford, Mechanical factors in distribution of pulmonary ventilation, *J. Appl. Physiol.* 8 (1956) 427–443 (<http://www.ncbi.nlm.nih.gov/pubmed/13286206> accessed August 7, 2015).
- [51] Z. Hantos, B. Daróczy, B. Suki, S. Nagy, J.J. Fredberg, Input impedance and peripheral inhomogeneity of dog lungs, *J. Appl. Physiol.* 72 (1992) 168–178 (<http://www.ncbi.nlm.nih.gov/pubmed/1537711> accessed September 27, 2015).
- [52] R.S. Carvalho, A.G.M. de Pinho, A.P.A. de Andrade, C.A.M.E. Silva, C.E. Gaio, P. Tavares, Analysis of pulmonary mechanics in an experimental model of sepsis., *J. Bras. Pneumol. Publicação Of. Da Soc. Bras. Pneumol. E Tisiologia.* 32 316–21. <http://www.ncbi.nlm.nih.gov/pubmed/17268731> (accessed August 11, 2015).