



# Inhibition of antigen-specific immune responses by co-application of an indoleamine 2,3-dioxygenase (IDO)-encoding vector requires antigen transgene expression focused on dendritic cells

Stephan Sudowe<sup>1,4</sup> · Yvonne Höhn<sup>1</sup> · Andrea Renzing<sup>1</sup> · Joachim Maxeiner<sup>2</sup> · Evelyn Montermann<sup>1</sup> · Alice Habermeier<sup>3</sup> · Ellen Closs<sup>3</sup> · Matthias Bros<sup>1</sup> · Angelika B. Reske-Kunz<sup>1</sup>

Received: 7 June 2019 / Accepted: 10 January 2020 / Published online: 1 February 2020  
© The Author(s) 2020

## Abstract

We have previously shown that particle-mediated epidermal delivery (PMED) of plasmids encoding  $\beta$ -galactosidase ( $\beta$ Gal) under control of the fascin-1 promoter (pFascin- $\beta$ Gal) yielded selective production of the protein in skin dendritic cells (DCs), and suppressed Th2 responses in a mouse model of type I allergy by inducing Th1/Tc1 cells. However, intranasal challenge of mice immunized with pFascin- $\beta$ Gal induced airway hyperreactivity (AHR) and neutrophilic inflammation in the lung. The tryptophan-catabolizing enzyme indoleamine 2,3-dioxygenase (IDO) has been implicated in immune suppression and tolerance induction. Here we investigated the consequences of co-application of an IDO-encoding vector on the modulatory effect of DNA vaccination by PMED using pFascin- $\beta$ Gal in models of eosinophilic allergic and non-eosinophilic intrinsic airway inflammation. IDO-encoding plasmids and pFascin- $\beta$ Gal or pCMV- $\beta$ Gal were co-applied to abdominal skin of BALB/c mice without, before or after sensitization with  $\beta$ Gal protein. Immune responses in the lung were analysed after intranasal provocation and airway reactivity was determined by whole body plethysmography. Co-application of pCMV-IDO with pFascin- $\beta$ Gal, but not pCMV- $\beta$ Gal inhibited the Th1/Tc1 immune response after PMED. Moreover, AHR in those mice was attenuated following intranasal challenge. Therapeutic vaccination of  $\beta$ Gal-sensitized mice with pFascin- $\beta$ Gal plus pCMV-IDO slightly suppressed airway inflammation and AHR after provocation with  $\beta$ Gal protein, while prophylactic vaccination was not effective. Altogether, our data suggest that only the combination of DC-restricted antigen and ubiquitous IDO expression attenuated asthma responses in mice, most probably by forming a tryptophan-depleted and kynurenine-enriched microenvironment known to affect neutrophils and T cells.

**Keywords** Indoleamine 2,3-dioxygenase (IDO) · DNA vaccination · Particle-mediated epidermal delivery (PMED) · Fascin promoter · Dendritic cells · Allergic airway inflammation

Handling Editor: L. V. Sinclair.

Stephan Sudowe, Yvonne Höhn, Matthias Bros, and Angelika B. Reske-Kunz equally contributed as first and senior authors.

**Electronic supplementary material** The online version of this article (<https://doi.org/10.1007/s00726-020-02817-4>) contains supplementary material, which is available to authorized users.

✉ Matthias Bros  
mbros@uni-mainz.de

<sup>1</sup> Department of Dermatology, University Medical Center of the Johannes Gutenberg University Mainz, Mainz, Germany

<sup>2</sup> III. Medical Department, Asthma Core Facility, University Medical Center of the Johannes Gutenberg University Mainz, Mainz, Germany

## Abbreviations

AHR	Airway hyperreactivity
APC	Antigen presenting cell
BAL	Bronchoalveolar lavage
$\beta$ Gal	$\beta$ -Galactosidase
DC	Dendritic cell
IDO	Indoleamine 2,3-dioxygenase

<sup>3</sup> Department of Pharmacology, University Medical Center of the Johannes Gutenberg University Mainz, Mainz, Germany

<sup>4</sup> Present Address: Ganzimmun Diagnostics AG, Mainz, Germany

PMED	Particle-mediated epidermal delivery
Tc	Cytotoxic T cell
Th	T-helper cell
Treg	Regulatory T cell
Trp	Tryptophan

## Introduction

Dendritic cells (DCs) play a pivotal role in initiating and controlling immune responses. Under steady-state conditions, immature DCs impart immune tolerance by inducing T-cell apoptosis, T-cell anergy or the differentiation of regulatory T cells (Tregs) (Iberg et al. 2017). Under inflammatory conditions, DCs are activated to constitute highly potent antigen presenting cells (APCs), capable of inducing differentiation of naïve antigen-specific T cells into T-effector cells of various polarization (Devi and Anandasabapathy 2017). However, to prevent excessive immune responses, proinflammatory mediators such as interferons, TNF- $\alpha$ , TGF- $\beta$  and distinct TLR ligands can induce the expression of two homologous genes encoding indoleamine 2,3-dioxygenase (IDO), *Ido1* and *Ido2* (Van der Leek et al. 2017). Both enzymes catalyse the initial, rate-limiting step in the degradation of tryptophan (Trp), internalized from the extracellular microenvironment. Withdrawal of Trp from the local microenvironment blocks entry of activated T cells into the cell cycle and activates Tregs (Munn et al. 2005). Furthermore, IDO-triggered formation of Trp degradation products, termed kynurenines, was demonstrated to exert suppressive function by inducing T-cell anergy and apoptosis, and Treg expansion, the latter via binding to the aryl hydrocarbon receptor (Van der Leek et al. 2017). Thus, IDO expression by DCs is associated with peripheral tolerance and the induction of immunosuppression (Wu et al. 2018; Mellor et al. 2017). DCs themselves are not affected directly by deprivation of Trp and accumulation of kynurenines (Terness et al. 2002), but may be limited in their functional activity by Tregs (Dhainaut and Moser 2015).

Due to their immune-modulatory potential, DCs are in the focus of developing immunotherapeutic approaches aimed to combat various diseases such as cancer, autoimmunity or allergies (Constantino et al. 2017). DCs transfected in vitro or in vivo with vectors encoding relevant antigen(s) either alone or in combination with coinhibitory/costimulatory molecules were successfully applied in various disease settings (Cheng et al. 2018). In vivo transfection of DCs with plasmid DNA employs intramuscular or intradermal needle injection or particle-mediated epidermal delivery (PMED) by gene gun, also termed biolistic transfection (Hohn et al. 2013). For the latter, plasmid DNA is coated onto micrometer-sized gold particles, and accelerated by helium pressure. We and others have shown

that PMED resulted in the transfection of keratinocytes and epidermal DCs, termed Langerhans cells, in human (Larregina et al. 2001) and mouse (Ross et al. 2003). In addition, Langerin<sup>+</sup> dermal DCs were identified as directly transfected in the course of PMED as well (Stoecklinger et al. 2011).

Using PMED, 10–100 times less DNA is needed to induce an effective immune response than when employing needle injection (Fynan et al. 1993). In most vaccination studies the strong and ubiquitously active CMV promoter has been used as the transgene regulatory unit (Williams 2013). In mouse models of type I allergy and of allergic airway inflammation, induced by subcutaneous injection of the model antigen  $\beta$ -galactosidase ( $\beta$ Gal) in alum, we used gene gun application of a plasmid encoding  $\beta$ Gal under control of the promoter of the gene encoding Fascin-1, a cytoskeletal F-actin-bundling protein (pFascin- $\beta$ Gal) (Raker et al. 2013; Stein et al. 2014). Fascin-1 is mandatory for the formation of dendrites by DCs as previously demonstrated by us (Ross et al. 1998), and is synthesized in high amounts by maturing and mature DCs. Fascin-1 is also expressed constitutively by neuronal and glial cells, but not by immature DCs and other types of immune cells (Yamashiro 2012). In accordance with PMED-induced DC activation, we have shown that the Fascin-1-promoter conferred transgene expression that was restricted to skin DCs (Ross et al. 2003). In allergy models, this DNA vaccination approach potently suppressed antigen-specific IgE immune responses, and was associated with a shift towards a T-helper cell type (Th)1-biased immune response and the recruitment of IFN- $\gamma$ -producing CD8<sup>+</sup> T cells both systemically (Sudowe et al. 2003, 2006) and locally in the lung following nasal provocation with the antigen (Zindler et al. 2008). Although DNA vaccination before sensitization reduces the number of eosinophils in the bronchoalveolar lavage (BAL), triggered by nasal  $\beta$ Gal-challenge, airway hyperreactivity (AHR) was not alleviated. This has been demonstrated to be due to the infiltration of neutrophils into the lung. Taken together, these data showed that the therapeutic conversion of the allergic Th2 response towards a Th1/cytotoxic T-cell type (Tc)1 response by PMED of an allergen-encoding plasmid has detrimental consequences by triggering pulmonary inflammatory reactions itself. Similarly, it has been reported that transfer of in vitro-differentiated T-cell receptor-transgenic Th1 cells induced the accumulation of neutrophils in the lungs of recipient mice and elevated AHR (Takaoka et al. 2001). We have subsequently demonstrated that PMED with pFascin- $\beta$ Gal qualifies as an experimental asthma model for non-eosinophilic airway inflammation and AHR (Stein et al. 2014). Interestingly, a subgroup of asthmatic patients suffers from non-atopic (intrinsic) asthma characterized by pulmonary accumulation of neutrophils, while eosinophils are almost absent (McGrath et al. 2012).

In this report, we analysed the suitability of IDO-encoding plasmids to generate tolerogenic DCs to inhibit the Th1/Tc1 response and ensuing neutrophilic pulmonary infiltration and AHR induced by PMED of a  $\beta$ Gal-encoding plasmid in models of non-eosinophilic intrinsic as well as eosinophilic allergic airway inflammation.

## Materials and methods

### Mice

Female BALB/c mice were bred and maintained under specific pathogen-free conditions on a standard diet in the Translational Animal Research Center of the University Medical Center Mainz.

### Plasmids

Construction and evaluation of pFascin- $\beta$ Gal, pCMV- $\beta$ Gal, pFascin-EGFP and CMV-EGFP were reported before (Ross et al. 2003; Sudowe et al. 2003). The coding region of murine IDO1 was amplified by PCR (sense primer: aagctagcagggttacagcgcctggcacacctggcct, anti-sense primer: aactcgagcttgctacactaaggccaactcagaagag) from total cDNA derived from LPS-stimulated bone marrow-derived DCs. pCMV-IDO was obtained by cloning the PCR product via the incorporated *NheI* and *XhoI* recognition sites into the CMV promoter containing expression vector pCI (Promega, Madison, MA), followed by internal deletion of the  $\beta$ -globin/IgG chimeric intron via *Bst98I* digest and relegation. pFascin-IDO was generated by exchange of the OVA expression cassette in pFascin-OVA by the IDO expression cassette derived from pCMV-IDO.

### In vitro analysis of expression constructs

NIH-3T3 cells were cultured in IMDM with 5% fetal calf serum (FCS, PAA, Cölbe, Germany), 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin (all from Sigma-Aldrich, Deisenhofen, Germany), and 50  $\mu$ M  $\beta$ -mercaptoethanol (Roth, Karlsruhe, Germany). NIH-3T3 cells were transfected with pCMV-IDO and pFascin-IDO, respectively, using Fugene HD as recommended by the manufacturer (Roche, Mannheim). After 5 h, the supernatant was replaced by culture medium. 24 h later, the culture medium was replaced by medium with a defined concentration of Trp (25  $\mu$ M). The contents of Trp and kynurenines in culture supernatants were determined by HPLC.

## Determination of TRP and kynurenine contents

The content of L-tryptophan and L-kynurenine in supernatants of NIH-3T3 cells was quantified simultaneously by HPLC using 3-nitro-L-tyrosine as an internal standard as described before (Laich et al. 2002; Widner et al. 1997), except that the elution buffer was 15 mmol/l acetic acid-sodium acetate, pH 4.2, containing 5% acetonitrile (Rotisol<sup>R</sup> HPLC Ultra Gradient Grad). For separation, a reverse-phase xBridge<sup>TM</sup> Shield RP 18 5  $\mu$ m 4.6 mm  $\times$  150 mm column (Waters, Milford, MA, USA) was used without precolumns. The flow rate was 1.3 ml/min at 30°. Tryptophan was quantified by a fluorescence detector (Hitachi 8470, Ex 285 nm, Em 365 nm) and kynurenines were measured by a UV detector (DAD 3L-OU, Bischoff, Leonberg, Germany, 360 nm) in flow stream series connection. The HPLC pumps (Model 2250, Bischoff) were controlled by an Lc CaDI 22-14 (Bischoff). Sample collection was performed using a Triathlon autosampler (Spark, Emmen Netherlands). Injected volumes were 5–30  $\mu$ l. Bischoff MqDacq software was used for peak quantification.

### Real-time PCR

Expression of candidate mRNA species in immunomagnetically sorted CD11c<sup>+</sup> DCs and T-cell populations (CD4<sup>+</sup>, CD8<sup>+</sup>) was monitored by real-time PCR. For this, total RNA was isolated from the according cell populations using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and reverse-transcribed (iScript; Bio-Rad, Munich, Germany) as recommended. Primer pairs for the detection of IDO (sense: 5'-AAGGGCTTCTTCCTCGTCTC-3', anti-sense: 5'-AAA AACGTGTCTGGGTCCAC-3'), Foxp3, (5'-CTCATCCGA TGGGCCATCCTGGAA-3, 5'-TTCCAGGTGGCGGGG TGGTTTCTG-3') IL-10 (5'-CCAAGCCTTATCGGAAAT GA-3, 5'-TTTTACAGGGGAGAAATCG-3'), TGF- $\beta$  (5'-TTGCTTCAGCTCCACAGAGA-3, 5'-TGGTTGTAGAGG GCAAGGGC-3'), and of the house-keeping gene GAPDH (5'-CCATCACCATCTTCCAGGAG-3, 5'-TTTCTCGTG GTTACACACC-3') were purchased from Eurofins MWG Synthesis (Ebersberg, Germany). The performance of real-time PCR has been described (Bros et al. 2007).

### Immunization protocol

For subsequent immunization studies, female BALB/c mice used previously demonstrated to show a higher susceptibility towards OVA-induced allergic airway inflammation than male mice (Melgert et al. 2005). Mice were immunized three times in weekly intervals with each a total of 4  $\mu$ g plasmid DNA of the indicated constructs using the Helios<sup>TM</sup> gene gun (Bio-Rad, Munich, Germany) as previously described (Zindler et al. 2008). In case of immunization of mice with

two different constructs, DNA of the according vectors (each 2 µg) was mixed prior to adsorption onto gold particles to ensure co-delivery of both plasmids into the same cell and hence its cotransfection. In some experiments, mice were sensitized by subcutaneous injection of 10 µg recombinant β-galactosidase (βGal; Sigma-Aldrich, Steinheim, Germany) dissolved in 200 µl of PBS (Conrad et al. 2009) either prior to or after immunization as indicated. Seven to ten days after the last treatment, anaesthetised mice were challenged by intranasal instillation of each 50 µl βGal protein (1 mg/ml in PBS) on three consecutive days (Zindler et al. 2008) as indicated.

### Measurement of airway function

One day after the last intranasal challenge with βGal, airway reactivity of differentially treated mice was monitored by non-invasive single-chamber whole-body barometric plethysmography (PLY 3211; Buxco Electronics, Birmingham, UK) as described (Raker et al. 2013). For this, mice were placed in a plethysmograph, and breathing-dependent changes in the body volume were measured (Jensen-Jarolim et al. 2017). The enhanced pause (Penh) response of non-anaesthetised mice reflects changes of the measured box pressure signal in waveform due to bronchoconstriction. The Penh baseline was assessed after inhalation of PBS and after exposure to increasing doses of aerosolized methacholine that induces bronchoconstriction was recorded for 5 min.

### Bronchoalveolar lavage

One day after measurement of airway reactivity, characterization of cells in the bronchoalveolar lavage (BAL) was performed as described (Zindler et al. 2008).

### Purification of immune cell populations

CD11c<sup>+</sup> DC, CD4<sup>+</sup> and CD8<sup>+</sup> T-cell populations were isolated from splenocytes and lymph node cell populations by immunomagnetic separation as recommended by the manufacturer (Miltenyi Biotec, Bergisch-Gladbach, Germany). The purity of the isolated cell populations was evaluated by flow cytometric analysis.

### Flow cytometry

Immunomagnetically sorted cells (10<sup>6</sup>) were washed with PBS/2% FCS and incubated with CD16/32-specific mAb 2.4.G2 (0.25 µg, rat IgG2b, purified from culture supernatants of hybridoma 2.4.G2) to avoid unspecific binding. Cells were then treated with the following primary mAb: PE/Cy5-conjugated anti-CD4 (clone GK1.5, Miltenyi Biotec, Bergisch Gladbach, Germany), PE- and Cy5/PE-conjugated

anti-CD8 (clone 53-6.7, Miltenyi Biotec), PE-conjugated anti-Foxp3 (clone 3G3, Miltenyi Biotec), and FITC-conjugated anti-CD25 (clone PC61.5.3, Caltag Burlingame, CA). Appropriate isotype controls were employed in parallel reactions. Cells were analysed using a FACS Canto II (BD Biosciences) equipped with DIVA Software.

### Cytokine quantification

Axillary and inguinal lymph node cells (5 × 10<sup>6</sup> cells) were cultured on 24-well tissue culture plates (Corning Costar, Bodenheim, Germany) in a volume of 1 ml culture medium (Iscove's Modified Dulbecco's Medium [Gibco, Paisley, UK] supplemented with 5% FCS [PAN Systems, Nürnberg, Germany], 50 µM 2-mercaptoethanol [Carl Roth GmbH & Co., Karlsruhe, Germany], 2 mM L-glutamine and 100 U/ml penicillin/100 µg/ml streptomycin [Gibco] with or without βGal (25 µg/ml). After 72 h, culture supernatants were collected and stored at -20 °C until quantification of the cytokine contents (IFN-γ, IL-5) by sandwich ELISA as described (Sudowe et al. 2003; Zindler et al. 2008).

### ELISPOT assay

Activated IFN-γ producing CD8<sup>+</sup> T cells specific for the H-2L<sup>d</sup>-binding βGal-derived nonamer peptide TPHPARIGL (Sigma-Aldrich) among single cell suspensions of draining axillary and inguinal lymph nodes were determined using an enzyme-linked immunosorbent spot-forming cell (ELISPOT) assay as described (Sudowe et al. 2003). Spots were enumerated using the ELISpot Reader System (AID, Strasbourg, Germany).

### Antibody titers in sera

βGal-specific IgG1 and IgG2a were determined as described (Sudowe et al. 2002). The antibody titer was defined as the reciprocal serum dilution yielding an absorbance reading of OD = 0.2 after linear regression analysis.

### Statistical analysis

Statistical tests were performed using SigmaPlot12.3 (Systat Software GmbH, Erkrath, Germany). Antibody titers and IFN-γ levels in sera, and frequencies of IFN-γ producing CD8<sup>+</sup> T cells derived from lymph nodes of mice differentially co-transfected with β-Gal-encoding plasmids and control vector or IDO-encoding vectors (pCMV-IDO, pFascin-IDO) as well as the Penh of differentially treated mice were assessed by Tukey two-way ANOVA. *p* values below 0.05 were considered statistically significant. All other data were analysed by Tukey one-way ANOVA.

## Results and discussion

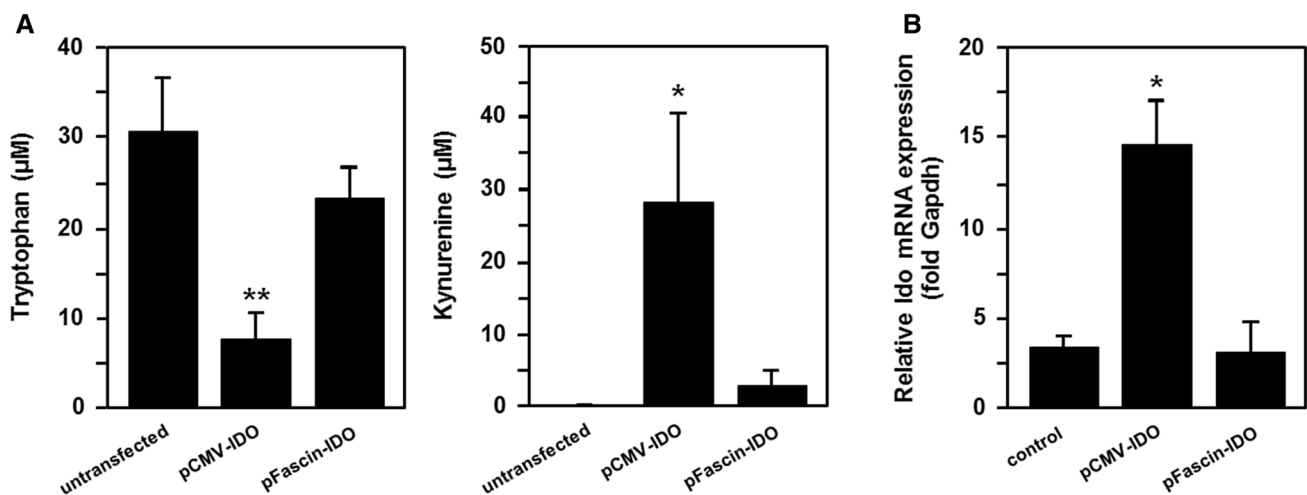
### IDO-encoding plasmid DNA is expressed in vitro and in vivo

This study aimed to assess the potency of IDO-encoding plasmids in combination with antigen-encoding vectors to generate tolerance-promoting DCs *in vivo* that inhibit inflammatory infiltration into the lung and AHR as induced in models of non-eosinophilic and eosinophilic allergic airway inflammation. We used vectors encoding  $\beta$ Gal under control of the fascin-1 promoter (pFascin- $\beta$ Gal) and the CMV promoter (pCMV- $\beta$ Gal), respectively, in combination with plasmids encoding IDO under control of either promoter (pFascin-IDO, pCMV-IDO). In these experiments, pFascin-EGFP encoding EGFP under control of the fascin-1 promoter served as a control vector.

The two isoforms of the rate-limiting enzyme in Trp degradation in most cell types, namely IDO-1 and IDO-2, are structurally similar, but differ in their expression pattern and signaling function (Pantouris et al. 2014). IDO-1 is expressed in many more cell types than IDO-2, and its immune function has been extensively studied. We, therefore, used the IDO-1 gene to construct pFascin-IDO and pCMV-IDO. The murine fibroblast cell line NIH-3T3 has been previously shown to express Fascin-1 (Jayo et al. 2016) indicating activity of the endogenous fascin-1

gene promoter. In contrast to DCs, this cell line can be transfected efficiently *in vitro*. To assess the enzymatic activity of IDO, transfected NIH-3T3 cells were cultured with 25  $\mu$ M Trp for 48 h, and the amounts of Trp and kynurenines in cell culture supernatants were determined by HPLC. Trp levels were strongly reduced in supernatants of cells transfected with pCMV-IDO as compared with the non-transfected control (Fig. 1a, left panel). This was not the case for supernatants of cells transfected with pFascin-IDO. Whilst kynurenines were hardly detectable in supernatants of non-transfected cells, supernatants from cells transfected with pCMV-IDO contained considerable concentrations of kynurenines, indicating functional activity of the transfected IDO (Fig. 1a, right panel). In supernatants from cells transfected with pFascin-IDO, this effect was much less pronounced. This discrepancy may be explained by a higher level activity of the CMV promoter as compared with the Fascin 1 gene promoter (Ross et al. 2003; Castor et al. 2018).

To analyse the capacity of the IDO-encoding constructs to evoke IDO expression by primary DCs *in vivo*, mice were transfected by PMED with pCMV-IDO, pFascin-IDO, or control vector on 5 non-overlapping regions of the shaved abdomen. After 2 days, inguinal and axillary lymph nodes were recovered and CD11c<sup>+</sup> DCs were isolated by immunomagnetic sorting. Real-time PCR revealed strongly enhanced IDO transcript levels in DCs isolated from mice transfected



**Fig. 1** Functional analysis of pCMV-IDO and pFascin-IDO *in vitro* and *in vivo*. **a** NIH-3T3 cells were transfected with pCMV-IDO or pFascin-IDO, each in combination with pCMV-EGFP to visualize transfection efficiency by fluorescence microscopy, using Fugene HD, or were mock transfected. After 1 day, medium was exchanged by culture medium containing Trp at defined concentration (25  $\mu$ M). After 48 h, the concentrations of Trp (left panel) and kynurenines (right panel) in cell culture supernatants were determined by HPLC. Data denote the mean  $\pm$  SEM of a total of four independent experiments each. **b** BALB/c mice were biolistically transfected with

pCMV-IDO, pFascin-IDO and with control vector on 5 non-overlapping regions of the shaved abdomen. After 2 days, inguinal and axillary lymph nodes were recovered, pooled, and CD11c<sup>+</sup> DCs were isolated by immunomagnetic sorting. IDO mRNA expression in CD11c<sup>+</sup> DCs was assessed by real-time PCR. Data denote relative IDO mRNA expression normalized to expression of the house-keeping gene GAPDH in each sample (mean  $\pm$  SEM of a total of three independent experiments). **a**, **b** Statistically significant differences versus the accordance level in **a** untreated samples, and **b** mice transfected with the control vector are indicated. \* $p$  < 0.05, \*\* $p$  < 0.01



with pCMV-IDO in comparison with DCs obtained from mice transfected with the control vector (Fig. 1b). In contrast, DCs obtained from pFascin-IDO-transfected mice did not express enhanced levels of IDO mRNA. However, the finding that cell-specific promoters induced lower protein expression than ubiquitously active promoters was reported also by others (Vandermeulen et al. 2009).

### Modulation of the $\beta$ Gal-specific immune response by co-application of IDO-encoding plasmids

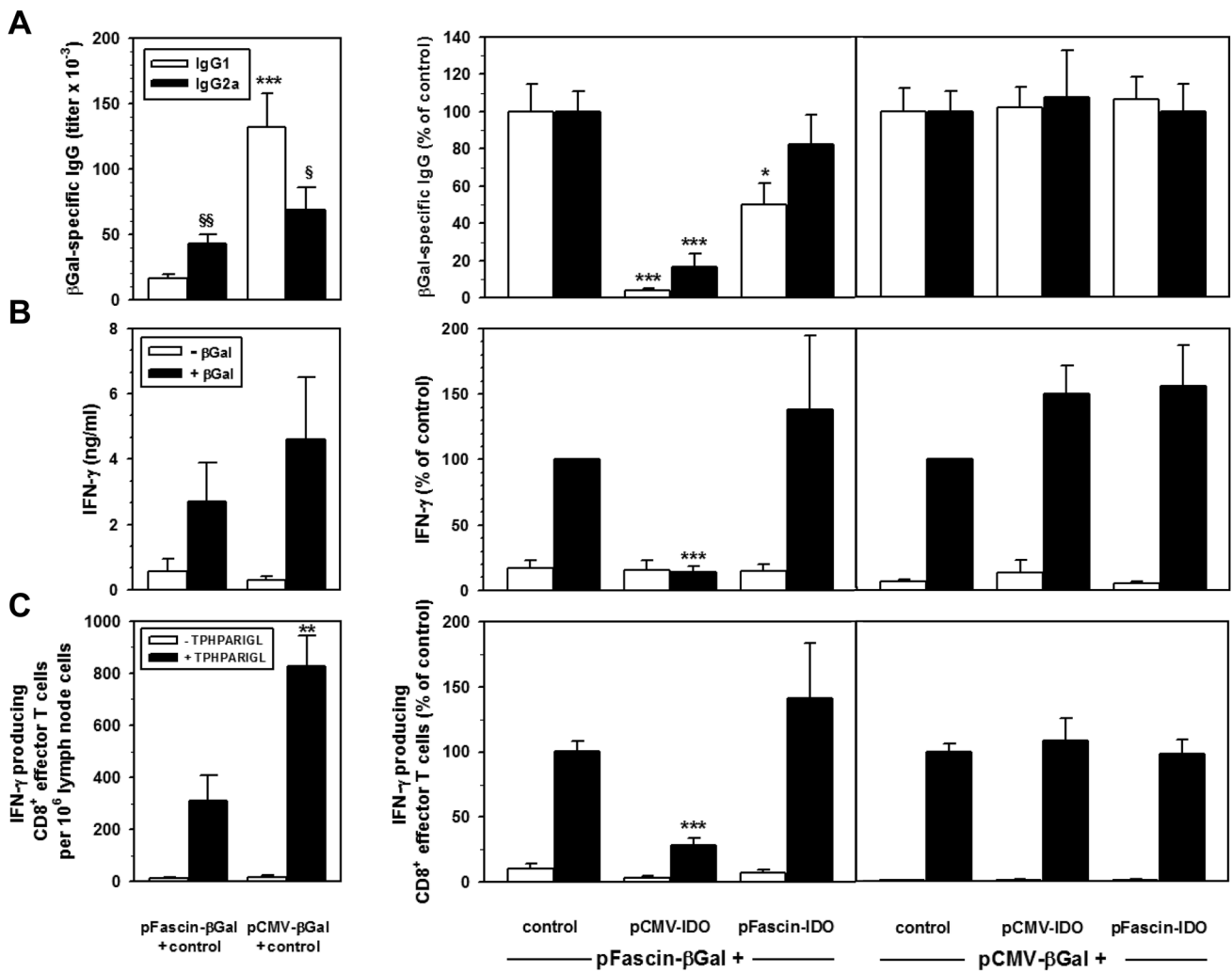
The  $\beta$ Gal- and the IDO-encoding plasmids or the control plasmid were mixed and co-adsorbed onto gold particles to achieve cotransfection of cells with both plasmids. We hypothesized that after PMED using pFascin- $\beta$ Gal, presentation of processed  $\beta$ Gal-peptides would be restricted to directly transfected DCs which are activated in the course of transfection (Larregina et al. 2001). When, on the other hand, pCMV- $\beta$ Gal is employed, the majority of transfected cells would be keratinocytes (Sudowe et al. 2009). It has previously been shown that apoptotic bodies derived from dying cells are internalized by DCs, and that these derived antigens may be presented via MHCII (Inaba et al. 1998), and may even be cross-presented via MHCI (Blachere et al. 2005). In agreement, we have demonstrated that biolistic vaccination with  $\beta$ -Gal-encoding vectors driven either by the keratinocyte-specific K5 or by the DC-specific Fascin 1 gene promoter resulted in similar T-cell activation (Sudowe et al. 2009). In this study, we demonstrated that strong induction of  $\beta$ -Gal-specific T-cell responses, where  $\beta$ Gal expression was restricted to keratinocytes, was mediated by the transfer of  $\beta$ Gal antigen to cutaneous DCs. Thus, it is plausible that the same mechanism of antigen transfer occurs in case of vaccination with CMV- $\beta$ -Gal due to the ubiquitous activity of the CMV promoter (Ross et al. 2003). Keratinocyte-derived microvesicles containing  $\beta$ -Gal are then internalized by adjacent (non-transfected) cutaneous DCs present in the epidermis. Since PMED activates DCs, presentation of  $\beta$ Gal-derived antigen of exogenous origin by DC—in the absence of IDO expression—may rather activate T cells.

Next, we analysed the potency of IDO-encoding plasmids to suppress the PMED-induced  $\beta$ Gal-specific immune response. For this, mice were immunized three times in weekly intervals by PMED using either pFascin- $\beta$ Gal or pCMV- $\beta$ Gal, each in combination with pCMV-IDO, pFascin-IDO or pFascin-EGFP as a control. After 1 week, mice were killed and the serum levels of  $\beta$ Gal-specific IgG1 and IgG2 were determined by ELISA. As reported before (Sudowe et al. 2006), immunization of mice with pFascin- $\beta$ Gal + control plasmid yielded lower levels of  $\beta$ -Gal-specific

IgG1 and IgG2a antibodies than immunization with pCMV- $\beta$ Gal + control vector (Fig. 2a, left panel). Furthermore, pCMV- $\beta$ Gal + control-vaccinated mice displayed higher titers of IgG1 than IgG2a, whilst the reverse was observed for mice transfected with pFascin- $\beta$ Gal (Fig. 2a, left panel). This finding is in accordance with our previous observations that antigen expression under the control of the Fascin-1 promoter induced Th1-biased humoral immune responses (Sudowe et al. 2006). Aside from activation of DCs by PMED (Larregina et al. 2001), the Th1 bias in response to DNA vaccination may also be explained in part by intrinsic adjuvancy of plasmids that contain CpG-rich elements, located, e.g. within the ampicillin resistance gene as well as promoter sequences that may trigger endo-/lysosomal TLR9 (Tudor et al. 2005) and cytosolic DNA-binding receptors (Suschak et al. 2016). However, our studies also demonstrated that the stimulatory effect of PMED and the intrinsic adjuvancy of DNA vaccines was overcome by co-transfection with plasmids that encoded tolerance-promoting cytokines like IL-10 and TGF- $\beta$  (Castor et al. 2018). Similarly, co-application of pFascin- $\beta$ Gal and pCMV-IDO resulted in significantly reduced serum titers of both Ig isotypes as compared with control mice treated with pFascin- $\beta$ Gal + control plasmid (Fig. 2a, right panel). The reduction in the levels of both isotypes was less pronounced, when pFascin-IDO was co-applied with pFascin- $\beta$ Gal. When pCMV- $\beta$ Gal was used for immunization, neither co-application of pCMV-IDO nor pFascin-IDO inhibited Ig production.

In vitro, antigen-stimulated IFN- $\gamma$  production was detected in culture supernatants of lymph node cells recovered from mice 13 days after the last immunization with pFascin- $\beta$ Gal and pCMV- $\beta$ Gal, respectively, co-administered with the control vector (Fig. 2b, left panel), whereas the levels of IL-5 were very low (data not shown). Together this indicates that PMED predominantly induced a Th1-response, as indicated by IFN- $\gamma$  production, rather than a Th2-response, as indicated by the lack of IL-5. In pFascin- $\beta$ Gal-treated mice, co-application of pCMV-IDO, but not of pFascin-IDO, completely inhibited IFN- $\gamma$  production (Fig. 2b, right panel). In the pCMV- $\beta$ Gal-treated mice, co-administration of pCMV-IDO did not exert an inhibitory effect on IFN- $\gamma$  production (Fig. 2b, right panel).

The number of  $\beta$ Gal peptide-induced IFN- $\gamma$ -producing CD8<sup>+</sup> effector T cells in draining lymph nodes, as determined by ELISPOT, was higher in mice immunized with pCMV- $\beta$ Gal than in those immunized with pFascin- $\beta$ Gal (Fig. 2c, left panel). In mice co-treated with pFascin- $\beta$ Gal and pCMV-IDO, but not with pFascin-IDO, the number of CD8<sup>+</sup> cells was reduced. This was not the case for pCMV- $\beta$ Gal-treated mice.



**Fig. 2** Co-application of pFascin-βGal and pCMV-IDO strongly attenuates βGal-specific Th1/Th2/Tc1 responses. BALB/c mice (4 mice each/group and experiment) were immunized three times in weekly intervals by gene gun-mediated transfection of pFascin-βGal or pCMV-βGal, each in combination with control vector (left panels) or in combination with control vector, pCMV-IDO or pFascin-IDO (right panels) as indicated (each 4 μg plasmid DNA/mouse per immunization). **a** One week after the third immunization, blood was retrieved, and levels of βGal-specific antibodies (IgG1, IgG2) in serum were determined by ELISA. **b, c** 13 days after the third immunization, axillary and inguinal lymph node cells were pooled, and

cultured in parallel for 3 days without or in the presence of **b** βGal protein (25 μg/ml) or **c** βGal-derived peptide (1 μg/ml). **b** Levels of IFN-γ in cell culture supernatants were assessed by ELISA. **c** The numbers of IFN-γ producing CD8<sup>+</sup> T cells were determined by ELISPOT assay. **a–c** Data denote the mean ± SEM of 20 mice compiled from five independent experiments. Statistically significant differences between IgG1 and IgG2a antibody titers (§), between control mice immunized with either pFascin-βGal or pCMV-βGal (\*), and between control mice and mice immunized with IDO-encoding plasmids (+) are indicated. §+ *p* < 0.05, §§+\* *p* < 0.01, \*\*\*+\*+ *p* < 0.001

Together, our data suggest that only in case of DC-restricted (pFascin-βGal) but not upon ubiquitous (pCMV-βGal) transgene expression (pCMV-βGal) tolerance may be induced. This requires ubiquitous co-expression of IDO (pCMV-IDO) to create a tolerance-promoting microenvironment. Induction of IDO expression in non-myeloid cells (DCs, macrophages and epithelial cells, especially in the lung) has been previously demonstrated in response

to systemic application of a CpG-rich immunostimulatory oligonucleotide serving as a TLR9 ligand (Hayashi et al. 2004).

Our data indicated that co-application of pCMV-IDO with a βgal antigen-encoding vector pFascin-βGal was superior to co-application with pFascin-IDO with regard to inhibition of IFN-γ-producing T cells. We, therefore, focused on pCMV-IDO in further experiments.

## Modulation of $\beta$ Gal-induced non-eosinophilic airway inflammation by biolistic co-application of $\beta$ Gal- and IDO-encoding plasmids

In most cases, asthma patients are atopic and present with IgE-mediated allergic asthma in response to sensitization and subsequent provocation with allergens (Daubeuf and Frossard 2014). In this, allergen-specific Th2 cells, IgE and other Th2-associated antibodies as well as strong lung infiltration of eosinophils play a central role. In mouse, eosinophilic asthma is mimicked by sensitization using a relevant antigen/allergen w/o or in combination with an adjuvant, followed by local challenge with the same antigen/allergen in the absence of any adjuvant (Yu and Chen 2018). A minor fraction of asthmatic patients are non-atopic, characterized by strong lung infiltrations of neutrophils instead of eosinophils, and in contrast to the former group are largely resistant towards treatment with glucocorticoids. We have previously shown that PMED of allergen-encoding pDNA yielded a non-atopic asthma phenotype in case allergen expression was confined to DCs using the Fascin-1 promoter (Sudowe et al. 2003, 2009).

Therefore, next we analysed, whether co-application of pCMV-IDO and  $\beta$ Gal-encoding vectors exerts an inhibitory effect on the antigen-elicited influx of neutrophils into the lung and on AHR. For this, mice were transfected three times with the different plasmid combinations by PMED, and were then challenged intranasally with  $\beta$ Gal protein on three consecutive days. Mice immunized with pFascin- $\beta$ Gal in combination with the control EGFP-encoding vector and subsequently provoked with  $\beta$ Gal exhibited extensive AHR after methacholine treatment as compared with mice challenged with PBS (Fig. 3a, left panel). However, co-application of pCMV-IDO in the course of vaccination prior to challenge resulted in considerably reduced AHR. In contrast, in the case of pCMV- $\beta$ Gal-immunized mice, co-administration of pCMV-IDO had no significantly attenuating effect on AHR (Fig. 3a, right panel).

We observed strong infiltration of neutrophils into the airway lumen of  $\beta$ Gal-challenged mice after PMED with pFascin- $\beta$ Gal (Fig. 3b, upper panel) and pCMV- $\beta$ Gal (Fig. 3b, lower panel), respectively, in case of co-application of the control vector, while the number of eosinophils in the bronchoalveolar lavage (BAL) was only slightly enhanced. Co-application of pCMV-IDO significantly inhibited the number of neutrophils in case of pFascin- $\beta$ Gal-treated mice. However, the reduction in neutrophil numbers in the group of pCMV- $\beta$ Gal-immunized mice, and IDO-mediated reduction in the numbers of lymphocytes in pFascin- and CMV- $\beta$ Gal-immunized mice were also not statistically significant.

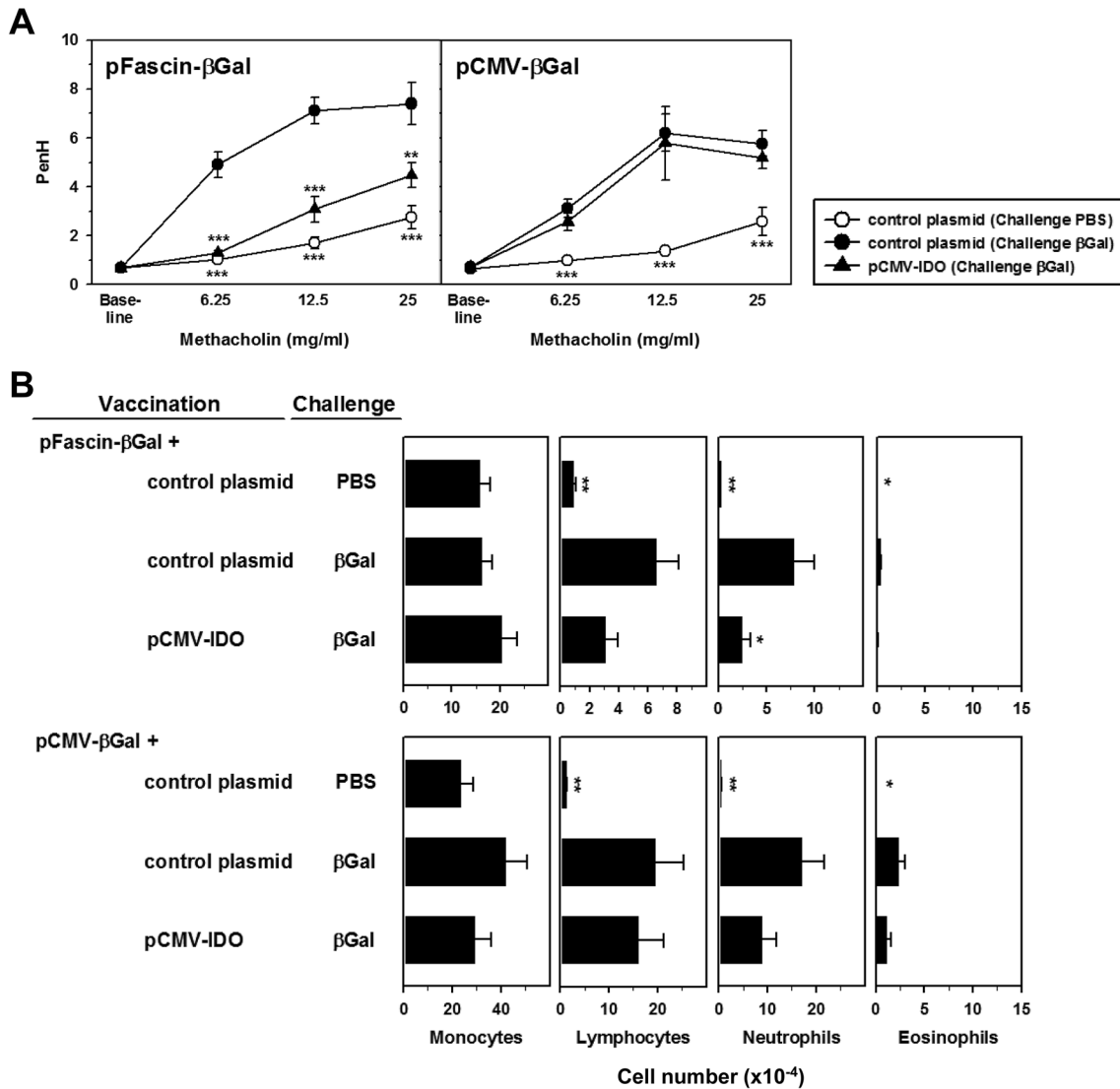
These results show that transfection-mediated ubiquitous expression of IDO only in context with DC-restricted (pFascin- $\beta$ Gal)  $\beta$ Gal presentation resulted in suppression of neutrophilic infiltration into the lung and considerable inhibition of AHR. This may be based on IDO-mediated deletion or functional inactivation (anergy induction) of  $\beta$ Gal-specific T cells or their differentiation towards Tregs (Mellor et al. 2017). Diminished T-cell activation would also impair chemokine production and concomitantly impair neutrophil attraction (Guida and Riccio 2019). In addition, kynurenins were reported to directly inhibit transendothelial migration of neutrophils (Loughman et al. 2016).

In further experiments we concentrated on the use of pFascin- $\beta$ Gal for immunization, because pCMV-IDO did not exert beneficial effects, when co-applied with pCMV- $\beta$ Gal.

## DC-focused biolistic co-application of $\beta$ Gal- and IDO-encoding plasmids does not affect the frequencies of Foxp3<sup>+</sup> regulatory T cells

IDO has been reported to exert its pro-tolerogenic effects via induction of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs (Mellor et al. 2017; Wu et al. 2018). So far, most studies have focused on the induction and functional properties of CD4<sup>+</sup> Treg (Klein et al. 2019). However, an increasing body of evidence has shown that CD8<sup>+</sup> T cells can differentiate to Foxp3<sup>+</sup> Treg as well (Yu et al. 2018). To assess the possibility that vaccination with CMV-IDO induced Foxp3<sup>+</sup> Treg, mice were vaccinated three times with pFascin- $\beta$ Gal together with the control plasmid or with pCMV-IDO. Naïve mice served as a control. One week after the last vaccination, the frequencies of Foxp3<sup>+</sup> Tregs in lymphoid organs were determined by flow cytometry. All three groups of mice had comparable frequencies of CD4<sup>+</sup> and CD8<sup>+</sup> Foxp3<sup>+</sup> Tregs in lymph nodes (Supplemental Fig. 2, left panel) and spleen (Supplemental Fig. 2, right panel). However, since the determination of Foxp3<sup>+</sup> frequency was performed antigen-unspecifically on the total T-cell populations, we cannot rule out subtle alterations in the frequencies of  $\beta$ -Gal-specific Tregs. This result is reminiscent of our previous finding of a failure to induce Foxp3<sup>+</sup> Tregs by biolistic vaccination of mice by PMED with a MOG-encoding vector in combination with plasmids encoding IL-10 or TGF- $\beta$ 1 which nonetheless resulted in an attenuated course of disease (Castor et al. 2018). Other induced Treg populations like Tr1 and Th3 cells are deficient for Foxp3, but mediate their suppressive effects via production of the anti-inflammatory cytokines IL-10 and TGF- $\beta$ , respectively (Ligocki and Niederkorn 2015). According to real-time PCR analysis, neither of these





**Fig. 3** Co-administration of pFascin-βGal and pCMV-IDO inhibits antigen-specific airway hyperresponsiveness. BALB/c mice were immunized three times in weekly intervals by gene gun-mediated transfection of pFascin-βGal or pCMV-βGal, each in combination with pCMV-IDO and control plasmid, respectively (see legend of Fig. 2). Ten days after the last immunization, mice were challenged by intranasal instillation of βGal protein (50 μg/ml) or PBS as control on three consecutive days. **a** One day later, airway reactivity was determined by non-invasive whole-body plethysmography, measuring

the enhanced pause (PenH) response both at baseline and after exposure to increasing doses of aerosolized methacholine. **b** Two days after the last application of βGal or PBS, lungs were flushed, isolated BAL cells (each  $5 \times 10^5$ ) were cytopspun, stained with DiffQuick, and quantitated. **a, b** Data denote the mean  $\pm$  SEM of 12 mice obtained in three independent experiments. Statistically significant differences versus the βGal-challenged control group are indicated. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$

cytokine mRNAs was differentially regulated which may be due to low frequencies of T cells with immuno-suppressive properties in the isolated CD4<sup>+</sup> and CD8<sup>+</sup> T-cell populations (Supplemental Fig. 3). Therefore, further studies are necessary to elucidate potential induction of Treg species in response to vaccination-induced IDO expression. Alternative mechanisms, including deletion of βGal-specific T cells,

or anergy induction in βGal-specific T cells interacting with DCs in a tryptophan-depleted micromilieu may account at least in part for the observed pro-tolerogenic outcome of vaccination. In a model of oral tolerance, anergy induction required administration of antigen at high dose, whereas active suppression resulted from feeding of antigen at low dose (Friedman and Weiner 1994).

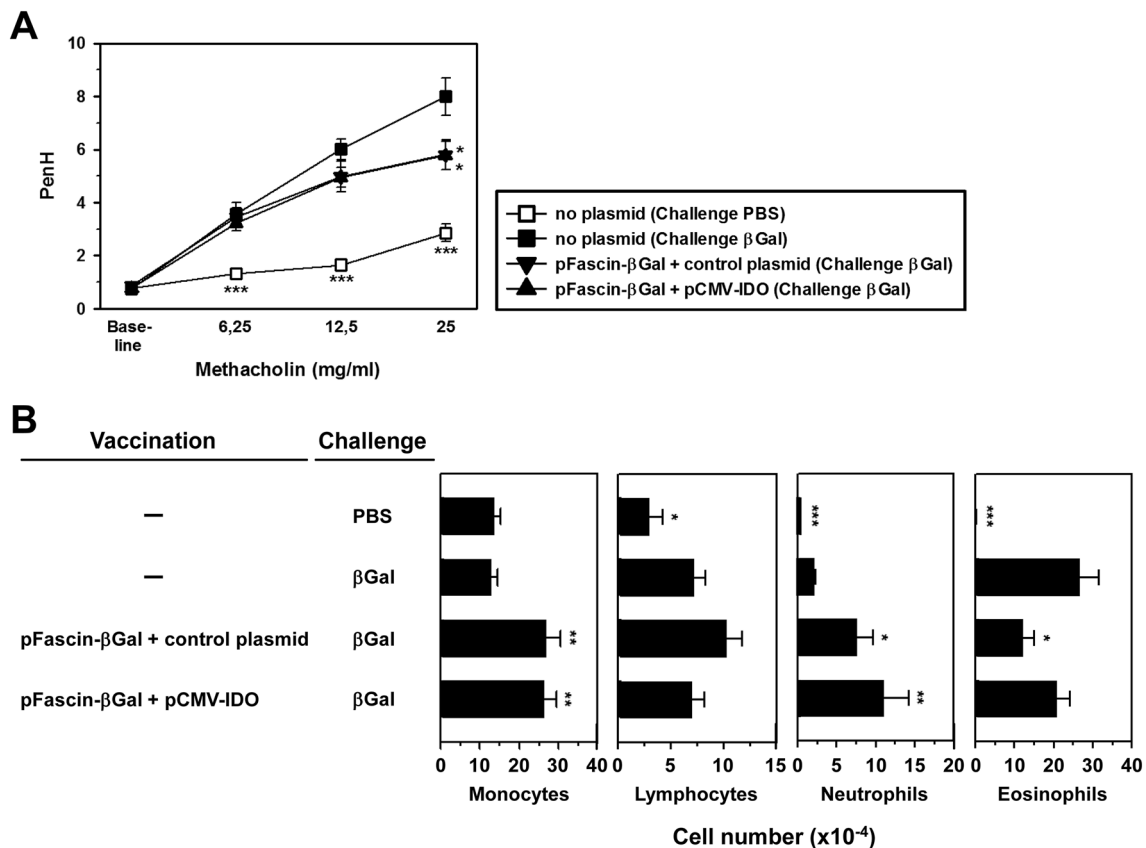
## Effect of DC-focused biolistic co-application of $\beta$ Gal- and IDO-encoding plasmids in a mouse model of allergic airway disease

Next we asked whether PMED with  $\beta$ Gal- and IDO-encoding vectors might exert an inhibitory effect on eosinophilic asthma induced by subsequent sensitization/provocation with  $\beta$ Gal protein as described above. To this end, mice were vaccinated three times by PMED and were then sensitized three times in weekly intervals by subcutaneous injection of  $\beta$ Gal protein (prophylactic vaccination). Eight days after the last sensitization, mice were intranasally challenged with  $\beta$ Gal protein on three consecutive days. Control mice, which did not receive PMED, but were sensitized with  $\beta$ Gal protein and challenged with  $\beta$ Gal protein developed a pronounced AHR as compared with mice challenged intranasally with PBS (Fig. 4a). PMED using pFascin- $\beta$ Gal in combination with the control plasmid resulted in slightly reduced AHR

after challenge with the highest methacholine dose. No further reduction in AHR was monitored when pCMV-IDO was co-applied instead of the control vector.

Control mice sensitized and challenged with  $\beta$ Gal experienced a moderate, but significant influx of lymphocytes and neutrophils and a strong infiltration of eosinophils into the lung lumen as compared with control mice that were challenged with PBS (Fig. 4b). Vaccination with PMED prior to  $\beta$ Gal-sensitization led to significantly increased infiltration of monocytes and neutrophils into the BAL. No significant difference was found in this respect between the groups co-treated with pCMV-IDO or the control vector. In the BAL of mice vaccinated with pFascin- $\beta$ Gal + control plasmid, we observed a significantly reduced influx of eosinophils, which was not seen with pFascin- $\beta$ Gal + pCMV-IDO-vaccinated mice.

Our results are in line with earlier findings showing that prophylactic vaccination with pFascin- $\beta$ Gal augmented lung



**Fig. 4** Prophylactic vaccination with pFascin- $\beta$ Gal and pCMV-IDO does not affect antigen-induced AHR. BALB/c mice (each 4 mice/group and experiment) were immunized three times in weekly intervals by gene gun-mediated transfection of pFascin- $\beta$ Gal in combination with the control plasmid or pCMV-IDO, respectively, or were left untreated. One week after the last immunization, mice were sensitized three times in weekly intervals by s.c. injection of  $\beta$ Gal protein (10  $\mu$ g). After 8 days, mice were intranasally provoked with  $\beta$ Gal-protein on three consecutive days. One group of control mice was

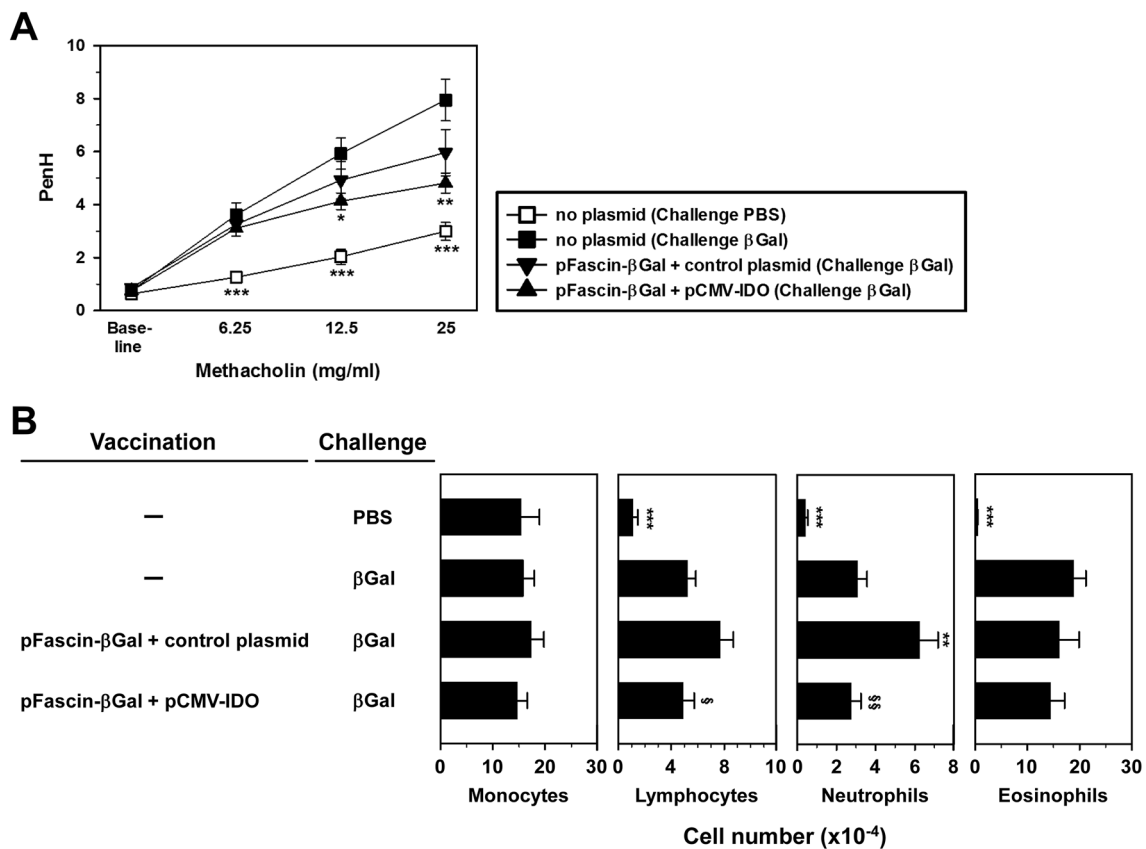
treated with PBS. **a** One day after the last application of  $\beta$ Gal protein or PBS, airway reactivity was determined and **b** 1 day later numbers of lung-infiltrating hematopoietic cell types were determined in BAL as described in the legend of Fig. 3. **a, b** Data denote the mean  $\pm$  SEM of 16 mice per group compiled from four independent experiments. Statistically significant differences versus the non-vaccinated control group challenged with  $\beta$ Gal protein are indicated. \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001

infiltration of neutrophils, concomitantly reduced numbers of infiltrating eosinophils, but conferred no substantial reduction of AHR (Zindler et al. 2008). Although ubiquitous expression of IDO in the context of DC-restricted  $\beta$ Gal-presentation had resulted in a substantial reduction of pulmonary neutrophils and of AHR tested directly following vaccination (see Fig. 3), this was not the case, when sensitization with  $\beta$ Gal protein was applied in tandem. This finding strengthens the notion that in a prophylactic setting, a tryptophan-depleted micromileu per se may not induce the differentiation of Tregs which would actively suppress symptoms of allergy.

In a further set of experiments, we analysed the therapeutic efficiency of co-applied pCMV-IDO in this mouse model of eosinophilic asthma disease. For this, mice were sensitized three times in weekly intervals by subcutaneous injection of  $\beta$ Gal protein. This induced a pronounced type

2 immune response as verified by high- $\beta$ Gal-specific IgG1 and low-IgG2a titers in sera (data not shown). Based on the IgG1 titers, mice were assigned to four groups, so that all cohorts exhibited a similar mean IgG1 titer. Two groups of mice were then subjected to PMED with pFascin- $\beta$ Gal together with pCMV-IDO and control plasmid, respectively. The remaining two groups were left untreated. When the latter two groups were intranasally challenged with  $\beta$ Gal and PBS, respectively, we observed pronounced AHR after the application of  $\beta$ Gal in contrast to treatment with PBS (Fig. 5a). After challenge of the two vaccinated groups of mice with  $\beta$ Gal, attenuated AHR was observed as compared with non-vaccinated mice. However, only co-application with pCMV-IDO resulted in a significant reduction in AHR.

In line with the differential outcome of AHR, the number of eosinophils, neutrophils and lymphocytes in BAL was



**Fig. 5** Therapeutic vaccination with pFascin- $\beta$ Gal and pCMV-IDO attenuates AHR. BALB/c mice (each 4 mice/group and experiment) were sensitized three times in weekly intervals by s.c. injection of  $\beta$ Gal or were left untreated. Five days after the last sensitization, titers of  $\beta$ Gal-specific antibodies were determined, and mice were grouped according to their individual IgG1 titer to obtain groups with similar means. After another week, two groups of mice were biologically transfected three times in weekly intervals with pFascin- $\beta$ Gal + control plasmid and pFascin- $\beta$ Gal + pCMV-IDO, respectively. Two weeks later, all mice were intranasally provoked with  $\beta$ Gal protein on

three consecutive days. **a** One day after the last application of  $\beta$ Gal protein or PBS, airway reactivity was determined and **b** 1 day later, numbers of lung-infiltrating hematopoietic cell types were determined in BAL as described in the legend of Fig. 3. **a, b** Data represent the mean  $\pm$  SEM of 8 (PMED with control plasmid) to 16 (no PMED, PMED with pCMV-IDO) mice per group compiled from four independent experiments. Statistically significant differences versus the non-immunized control group challenged with  $\beta$ Gal protein (\*) and versus the PMED group with control plasmid (§) are indicated. \* $\S$   $p < 0.05$ , \*\* $\S\S$   $p < 0.01$ , \*\*\* $p < 0.001$

enhanced in control mice challenged with  $\beta$ Gal versus PBS (Fig. 5b). In mice vaccinated with pFascin- $\beta$ Gal plus the control vector, similar numbers of eosinophils and lymphocytes, and significantly increased numbers of neutrophils were observed as compared with non-vaccinated mice challenged with  $\beta$ -Gal protein. In contrast, co-treatment of mice with pFascin- $\beta$ Gal and pCMV-IDO resulted in a significantly decreased influx of neutrophils and lymphocytes, to similar levels as observed for sensitized/challenged mice that had not been vaccinated.

Pulmonary influx of neutrophils was associated with the induction of Th1 (Yang et al. 2009) and Th17 cells (McKinley et al. 2008). In our model, CD4<sup>+</sup> and CD8<sup>+</sup> T cells were shown to cooperate to induce maximal neutrophilic infiltration (Stein et al. 2014). The finding that infiltration of neutrophils was significantly suppressed by co-application of pCMV-IDO following  $\beta$ Gal protein sensitization suggests that IDO (over)expression in transfected DCs may, on the one hand, via release of kynurenins, directly inhibit neutrophil migration and thereby their lung infiltration (Loughman et al. 2016). On the other hand, a tryptophan-depleted and kynurenine-rich microenvironment may affect the viability and migratory activity of antigen-specific T cells that engage antigen-presenting DC. This hypothesis is in line with reports that Trp-metabolites are toxic to CD4<sup>+</sup> Th1 cells (Xu et al. 2008) and CD8<sup>+</sup> T cells (Liu et al. 2014). IDO-mediated T-cell inhibition could explain the reduced numbers of lung-infiltrating lymphocytes although T cells and B cells were not differentiated in these measurements. Further studies are necessary to analyse in detail which cell type(s) are directly affected by IDO expression in this experimental setting.

In conclusion, our study revealed that DC-restricted antigen presentation as achieved using the fascin-1 promoter in combination with ubiquitous IDO expression is mandatory for successful immuno-intervention as reflected by suppressed generation of antigen-specific Th1 and Tc1 cells as well as AHR and neutrophilic airway inflammation after intranasal provocation with  $\beta$ Gal protein. However, in a model of allergic airway disease, this immuno-inhibitory effect was less pronounced, when PMED was employed as therapeutic vaccination, and was not observed upon prophylactic vaccination. Thus, our findings support that DC-restricted antigen expression, in combination with tryptophan depletion in the microenvironment, anergize or inhibit antigen-specific T cells and directly impair short-lived neutrophils rather than facilitate the induction of Treg. Further studies are necessary to delineate specific cellular mechanisms contributing to the tolerance-promoting effect of DC-restricted antigen presentation in the context of ubiquitous IDO activity.

**Acknowledgements** Open Access funding provided by Projekt DEAL. We thank Petra Schuster for expert technical assistance.

**Funding** This work was supported by the German Research Foundation, SFB 548. MB is funded by the SFB 1066(B05).

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** This study used experimental animals. The recommendations of the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health (NIH Publication No. 8023, revised 1978) were followed. The Ethics Commission according to the German Animal Welfare Act (Landesuntersuchungsamt of the state Rhineland-Palatinate, Reference no. 23 170-07/G07-1-023) approved the experiments in this study.

**Informed consent** Informed consent was obtained from all individual participants included in the study.

**Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

## References

- Blachere NE, Darnell RB, Albert ML (2005) Apoptotic cells deliver processed antigen to dendritic cells for cross-presentation. *PLoS Biol* 3(6):e185. <https://doi.org/10.1371/journal.pbio.0030185>
- Bros M, Jahrling F, Renzing A, Wiechmann N, Dang NA, Sutter A, Ross R, Knop J, Sudowe S, Reske-Kunz AB (2007) A newly established murine immature dendritic cell line can be differentiated into a mature state, but exerts tolerogenic function upon maturation in the presence of glucocorticoid. *Blood* 109(9):3820–3829. <https://doi.org/10.1182/blood-2006-07-035576>
- Castor T, Yogev N, Blank T, Barwig C, Prinz M, Waisman A, Bros M, Reske-Kunz AB (2018) Inhibition of experimental autoimmune encephalomyelitis by tolerance-promoting DNA vaccination focused to dendritic cells. *PLoS ONE* 13(2):e0191927. <https://doi.org/10.1371/journal.pone.0191927>
- Cheng MA, Farmer E, Huang C, Lin J, Hung CF, Wu TC (2018) Therapeutic DNA vaccines for human papillomavirus and associated diseases. *Hum Gene Ther*. <https://doi.org/10.1089/hum.2017.197>
- Conrad ML, Yildirim AO, Sonar SS, Kilic A, Sudowe S, Lunow M, Teich R, Renz H, Garn H (2009) Comparison of adjuvant and adjuvant-free murine experimental asthma models. *Clin Exp Allergy* 39(8):1246–1254. <https://doi.org/10.1111/j.1365-2222.2009.03260.x>
- Constantino J, Gomes C, Falcao A, Neves BM, Cruz MT (2017) Dendritic cell-based immunotherapy: a basic review and recent

- advances. *Immunol Res* 65(4):798–810. <https://doi.org/10.1007/s12026-017-8931-1>
- Daubeuf F, Frossard N (2014) Eosinophils and the ovalbumin mouse model of asthma. *Methods Mol Biol* (Clifton, NJ) 1178:283–293. [https://doi.org/10.1007/978-1-4939-1016-8\\_24](https://doi.org/10.1007/978-1-4939-1016-8_24)
- Devi KS, Anandasabapathy N (2017) The origin of DCs and capacity for immunologic tolerance in central and peripheral tissues. *Semin Immunopathol* 39(2):137–152. <https://doi.org/10.1007/s00281-016-0602-0>
- Dhainaut M, Moser M (2015) Mechanisms of surveillance of dendritic cells by regulatory T lymphocytes. *Progress Mol Biol Transl Sci* 136:131–154. <https://doi.org/10.1016/bs.pmbs.2015.08.003>
- Friedman A, Weiner HL (1994) Induction of anergy or active suppression following oral tolerance is determined by antigen dosage. *Proc Natl Acad Sci USA* 91(14):6688–6692
- Fynan EF, Webster RG, Fuller DH, Haynes JR, Santoro JC, Robinson HL (1993) DNA vaccines: protective immunizations by parental, mucosal, and gene-gun inoculations. *Proc Natl Acad Sci USA* 90(24):11478–11482
- Guida G, Riccio AM (2019) Immune induction of airway remodeling. *Semin Immunol*. <https://doi.org/10.1016/j.smim.2019.101346>
- Hayashi T, Beck L, Rossetto C, Gong X, Takikawa O, Takabayashi K, Broide DH, Carson DA, Raz E (2004) Inhibition of experimental asthma by indoleamine 2,3-dioxygenase. *J Clin Invest* 114(2):270–279. <https://doi.org/10.1172/jci21275>
- Hohn Y, Sudowe S, Reske-Kunz AB (2013) Dendritic cell-specific biolistic transfection using the fascin gene promoter. *Methods Mol Biol* (Clifton, NJ) 940:199–213. [https://doi.org/10.1007/978-1-62703-110-3\\_17](https://doi.org/10.1007/978-1-62703-110-3_17)
- Iberg CA, Jones A, Hawiger D (2017) Dendritic cells as inducers of peripheral tolerance. *Trends Immunol* 38(11):793–804. <https://doi.org/10.1016/j.it.2017.07.007>
- Inaba K, Turley S, Yamaide F, Iyoda T, Mahnke K, Inaba M, Pack M, Subklewe M, Sauter B, Sheff D, Albert M, Bhardwaj N, Mellman I, Steinman RM (1998) Efficient presentation of phagocytosed cellular fragments on the major histocompatibility complex class II products of dendritic cells. *J Exp Med* 188(11):2163–2173. <https://doi.org/10.1084/jem.188.11.2163>
- Jayo A, Malboubi M, Antoku S, Chang W, Ortiz-Zapater E, Groen C, Pfisterer K, Tootle T, Charras G, Gundersen GG, Parsons M (2016) Fascin regulates nuclear movement and deformation in migrating cells. *Dev Cell* 38(4):371–383. <https://doi.org/10.1016/j.devcel.2016.07.021>
- Jensen-Jarolim E, Pali-Scholl I, Roth-Walter F (2017) Outstanding animal studies in allergy I. From asthma to food allergy and anaphylaxis. *Curr Opin Allergy Clin Immunol* 17(3):169–179. <https://doi.org/10.1097/aci.0000000000000363>
- Klein L, Robey EA, Hsieh CS (2019) Central CD4(+) T cell tolerance: deletion versus regulatory T cell differentiation. *Nat Rev Immunol* 19(1):7–18. <https://doi.org/10.1038/s41577-018-0083-6>
- Laich A, Neurauter G, Widner B, Fuchs D (2002) More rapid method for simultaneous measurement of tryptophan and kynurenine by HPLC. *Clin Chem* 48(3):579–581
- Larregina AT, Watkins SC, Erdos G, Spencer LA, Storkus WJ, Beer Stolz D, Falo LD Jr (2001) Direct transfection and activation of human cutaneous dendritic cells. *Gene Ther* 8(8):608–617. <https://doi.org/10.1038/sj.gt.3301404>
- Ligocki AJ, Niederkorn JY (2015) Advances on non-CD4 + Foxp3+ T regulatory cells: CD8+, type 1, and double negative T regulatory cells in organ transplantation. *Transplantation* 99(8):1553–1559. <https://doi.org/10.1097/tp.0000000000000813>
- Liu WL, Lin YH, Xiao H, Xing S, Chen H, Chi PD, Zhang G (2014) Epstein-Barr virus infection induces indoleamine 2,3-dioxygenase expression in human monocyte-derived macrophages through p38/mitogen-activated protein kinase and NF-kappaB pathways: impairment in T cell functions. *J Virol* 88(12):6660–6671. <https://doi.org/10.1128/jvi.03678-13>
- Loughman JA, Yarbrough ML, Tiemann KM, Hunstad DA (2016) Local generation of kynurenines mediates inhibition of neutrophil chemotaxis by uropathogenic *Escherichia coli*. *Infect Immun* 84(4):1176–1183. <https://doi.org/10.1128/iai.01202-15>
- McGrath KW, Icitovic N, Boushey HA, Lazarus SC, Sutherland ER, Chinchilli VM, Fahy JV (2012) A large subgroup of mild-to-moderate asthma is persistently noneosinophilic. *Am J Respir Crit Care Med* 185(6):612–619. <https://doi.org/10.1164/rccm.201109-1640OC>
- McKinley L, Alcorn JF, Peterson A, Dupont RB, Kapadia S, Logar A, Henry A, Irvin CG, Piganelli JD, Ray A, Kolls JK (2008) TH17 cells mediate steroid-resistant airway inflammation and airway hyperresponsiveness in mice. *J Immunol* (Baltimore, Md: 1950) 181(6):4089–4097
- Melgert BN, Postma DS, Kuipers I, Geerlings M, Luinge MA, van der Strate BW, Kerstjens HA, Timens W, Hylkema MN (2005) Female mice are more susceptible to the development of allergic airway inflammation than male mice. *Clin Exp Allergy* 35(11):1496–1503. <https://doi.org/10.1111/j.1365-2222.2005.02362.x>
- Mellor AL, Lemos H, Huang L (2017) Indoleamine 2,3-dioxygenase and tolerance: where are we now? *Front Immunol* 8:1360. <https://doi.org/10.3389/fimmu.2017.01360>
- Munn DH, Mellor AL, Rossi M, Young JW (2005) Dendritic cells have the option to express IDO-mediated suppression or not. *Blood* 105(6):2618. <https://doi.org/10.1182/blood-2005-01-0122>
- Pantouris G, Serys M, Yuasa HJ, Ball HJ, Mowat CG (2014) Human indoleamine 2,3-dioxygenase-2 has substrate specificity and inhibition characteristics distinct from those of indoleamine 2,3-dioxygenase-1. *Amino Acids* 46(9):2155–2163. <https://doi.org/10.1007/s00726-014-1766-3>
- Raker V, Maxeiner J, Reske-Kunz AB, Sudowe S (2013) Efficiency of biolistic DNA vaccination in experimental type I allergy. *Methods Mol Biol* (Clifton, NJ) 940:357–370. [https://doi.org/10.1007/978-1-62703-110-3\\_26](https://doi.org/10.1007/978-1-62703-110-3_26)
- Ross R, Ross XL, Schwing J, Langin T, Reske-Kunz AB (1998) The actin-bundling protein fascin is involved in the formation of dendritic processes in maturing epidermal Langerhans cells. *J Immunol* (Baltimore, Md: 1950) 160(8):3776–3782
- Ross R, Sudowe S, Beisner J, Ross XL, Ludwig-Portugall I, Steitz J, Tuting T, Knop J, Reske-Kunz AB (2003) Transcriptional targeting of dendritic cells for gene therapy using the promoter of the cytoskeletal protein fascin. *Gene Ther* 10(12):1035–1040. <https://doi.org/10.1038/sj.gt.3301968>
- Stein J, Maxeiner JH, Montermann E, Hohn Y, Raker V, Taube C, Sudowe S, Reske-Kunz AB (2014) Non-eosinophilic airway hyper-reactivity in mice, induced by IFN-gamma producing CD4(+) and CD8(+) lung T cells, is responsive to steroid treatment. *Scand J Immunol* 80(5):327–338. <https://doi.org/10.1111/sji.12217>
- Stoecklinger A, Eticha TD, Mesdaghi M, Kissenfennig A, Malissen B, Thalhamer J, Hammerl P (2011) Langerin+ dermal dendritic cells are critical for CD8+ T cell activation and IgH gamma-1 class switching in response to gene gun vaccines. *J Immunol* (Baltimore, Md: 1950) 186(3):1377–1383. <https://doi.org/10.4049/jimmunol.1002557>
- Sudowe S, Montermann E, Steitz J, Tuting T, Knop J, Reske-Kunz AB (2002) Efficacy of recombinant adenovirus as vector for allergen gene therapy in a mouse model of type I allergy. *Gene Ther* 9(2):147–156. <https://doi.org/10.1038/sj.gt.3301625>
- Sudowe S, Ludwig-Portugall I, Montermann E, Ross R, Reske-Kunz AB (2003) Transcriptional targeting of dendritic cells in gene gun-mediated DNA immunization favors the induction of type I immune responses. *Mol Ther* 8(4):567–575



- Sudowe S, Ludwig-Portugall I, Montermann E, Ross R, Reske-Kunz AB (2006) Prophylactic and therapeutic intervention in IgE responses by biolistic DNA vaccination primarily targeting dendritic cells. *J Allergy Clin Immunol* 117(1):196–203. <https://doi.org/10.1016/j.jaci.2005.08.058>
- Sudowe S, Dominitzki S, Montermann E, Bros M, Grabbe S, Reske-Kunz AB (2009) Uptake and presentation of exogenous antigen and presentation of endogenously produced antigen by skin dendritic cells represent equivalent pathways for the priming of cellular immune responses following biolistic DNA immunization. *Immunology* 128(1 Suppl):e193–205. <https://doi.org/10.1111/j.1365-2567.2008.02947.x>
- Suschak JJ, Wang S, Fitzgerald KA, Lu S (2016) A cGAS-Independent STING/IRF7 pathway mediates the immunogenicity of DNA vaccines. *J Immunol* (Baltimore, Md: 1950) 196(1):310–316. <https://doi.org/10.4049/jimmunol.1501836>
- Takaoka A, Tanaka Y, Tsuji T, Jinushi T, Hoshino A, Asakura Y, Mita Y, Watanabe K, Nakaike S, Togashi Y, Koda T, Matsu-shima K, Nishimura T (2001) A critical role for mouse CXC chemokine(s) in pulmonary neutrophilia during Th type 1-dependent airway inflammation. *J Immunol* (Baltimore, Md: 1950) 167(4):2349–2353
- Terness P, Bauer TM, Rose L, Dufter C, Watzlik A, Simon H, Opelz G (2002) Inhibition of allogeneic T cell proliferation by indoleamine 2,3-dioxygenase-expressing dendritic cells: mediation of suppression by tryptophan metabolites. *J Exp Med* 196(4):447–457
- Tudor D, Dubuquoy C, Gaboriau V, Lefevre F, Charley B, Riffault S (2005) TLR9 pathway is involved in adjuvant effects of plasmid DNA-based vaccines. *Vaccine* 23(10):1258–1264. <https://doi.org/10.1016/j.vaccine.2004.09.001>
- Van der Leek AP, Yanishevsky Y, Kozyrskiy AL (2017) The Kynurenine pathway as a novel link between allergy and the gut microbiome. *Front Immunol* 8:1374. <https://doi.org/10.3389/fimmu.2017.01374>
- Vandermeulen G, Richiardi H, Escriou V, Ni J, Fournier P, Schirmacher V, Scherman D, Preat V (2009) Skin-specific promoters for genetic immunisation by DNA electroporation. *Vaccine* 27(32):4272–4277. <https://doi.org/10.1016/j.vaccine.2009.05.022>
- Widner B, Werner ER, Schennach H, Wachter H, Fuchs D (1997) Simultaneous measurement of serum tryptophan and kynurenine by HPLC. *Clin Chem* 43(12):2424–2426
- Williams JA (2013) Vector design for improved DNA vaccine efficacy, safety and production. *Vaccines* 1(3):225–249. <https://doi.org/10.3390/vaccines1030225>
- Wu H, Gong J, Liu Y (2018) Indoleamine 2, 3-dioxygenase regulation of immune response (review). *Mol Med Rep* 17(4):4867–4873. <https://doi.org/10.3892/mmr.2018.8537>
- Xu H, Zhang GX, Ciric B, Rostami A (2008) IDO: a double-edged sword for T(H)1/T(H)2 regulation. *Immunol Lett* 121(1):1–6. <https://doi.org/10.1016/j.imlet.2008.08.008>
- Yamashiro S (2012) Functions of fascin in dendritic cells. *Crit Rev Immunol* 32(1):11–21
- Yang M, Kumar RK, Foster PS (2009) Pathogenesis of steroid-resistant airway hyperresponsiveness: interaction between IFN-gamma and TLR4/MyD88 pathways. *J Immunol* (Baltimore, Md: 1950) 182(8):5107–5115. <https://doi.org/10.4049/jimmunol.0803468>
- Yu QL, Chen Z (2018) Establishment of different experimental asthma models in mice. *Exp Ther Med* 15(3):2492–2498. <https://doi.org/10.3892/etm.2018.5721>
- Yu Y, Ma X, Gong R, Zhu J, Wei L, Yao J (2018) Recent advances in CD8(+) regulatory T cell research. *Oncol Lett* 15(6):8187–8194. <https://doi.org/10.3892/ol.2018.8378>
- Zindler E, Gehrke N, Luft C, Reuter S, Taube C, Finotto S, Reske-Kunz AB, Sudowe S (2008) Divergent effects of biolistic gene transfer in a mouse model of allergic airway inflammation. *Am J Respir Cell Mol Biol* 38(1):38–46. <https://doi.org/10.1165/rcmb.2007-0067OC>

**Publisher's Note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.