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ORIGINAL ARTICLE

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

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Abstract

We have previously shown that particle-mediated epidermal delivery (PMED) of plasmids encoding β-galactosidase (βGal) under control of the fascin-1 promoter (pFascin-βGal) 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-βGal 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-βGal in models of eosinophilic allergic and non-eosinophilic intrinsic airway inflammation. IDO-encoding plasmids and pFascin-βGal or pCMV-βGal were co-applied to abdominal skin of BALB/c mice without, before or after sensitization with βGal 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-βGal, but not pCMV-βGal inhibited the Th1/Tc1 immune response after PMED. Moreover, AHR in those mice was attenuated following intranasal challenge. Therapeutic vaccination of βGal-sensitized mice with pFascin-βGal plus pCMV-IDO slightly suppressed airway inflammation and AHR after provocation with βGal 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 micromilieu 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

Abbreviations

AHR	Airway hyperreactivity
APC	Antigen presenting cell
BAL	Bronchoalveolar lavage
βGal	β-Galactosidase
DC	Dendritic cell
IDO	Indoleamine 2,3-dioxygenase

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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-α, TGF-β 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$^+$ 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 β-galactosidase (βGal) in alum, we used gene gun application of a plasmid encoding βGal under control of the promoter of the gene encoding Fascin-1, a cytoskeletal F-actin-bundling protein (pFascin-βGal) (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-γ-producing CD8$^+$ 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 βGal-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-βGal 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 βGal-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-βGal, pCMV-βGal, 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: aagctagcaggttacagcgcctggcacacctggccct, 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 *Nhe*I and *Xho*I recognition sites into the CMV promotor containing expression vector pCI (Promega, Madison, MA), followed by internal deletion of the β-globin/IgG chimeric intron via *Bst*98I 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 µg/ml streptomycin (all from Sigma-Aldrich, Deisenhofen, Germany), and 50 µM β-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 µM). 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 (Rotisolv^R HPLC Ultra Gradient Grad). For separation, a reverse-phase xBridge™ Shield RP 18 5 µm 4.6 mm × 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 mm) 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 µl. Bischoff MqDAcq software was used for peak quantification.

Real-time PCR

Expression of candidate mRNA species in immunomagnetically sorted CD11c$^+$ DCs and T-cell populations (CD4$^+$, CD8$^+$) 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′-AAAAACGTGTCTGGGTCCAC-3′), Foxp3, (5′-CTCATCCGATGGGCCATCCTGGAA-3, 5′-TTCCAGGTGGCGGGGTGGTTTCTG-3′) IL-10 (5′-CCAAGCCTTATCGGAAATGA-3, 5′-TTTTCACAGGGGAGAAATCG-3′), TGF-β (5′-TTGCTTCAGCTCCACAGAGA-3, 5′-TGGTTGTAGAGGGCAAGGGC-3′), and of the house-keeping gene GAPDH (5′-CCATCACCATCTTCCAGGAG-3, 5′-TTTCTCGTGGTTCACACCC-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 µg plasmid DNA of the indicated constructs using the Helios™ 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

$CD11c^+$ DC, $CD4^+$ and $CD8^+$ 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 (10^6) 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 Burlingham, 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 (5×10^6 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 $CD8^+$ T cells specific for the H-$2L^d$-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, Strasberg, 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 $CD8^+$ 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 βGal under control of the fascin-1 promoter (pFascin-βGal) and the CMV promoter (pCMV-βGal), 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 promotor 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 promotor. 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 µM 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 promotor (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$^+$ DCs were isolated by immunomagnetic sorting. Real-time PCR revealed strongly enhanced IDO transcript levels in DCs isolated from mice transfected

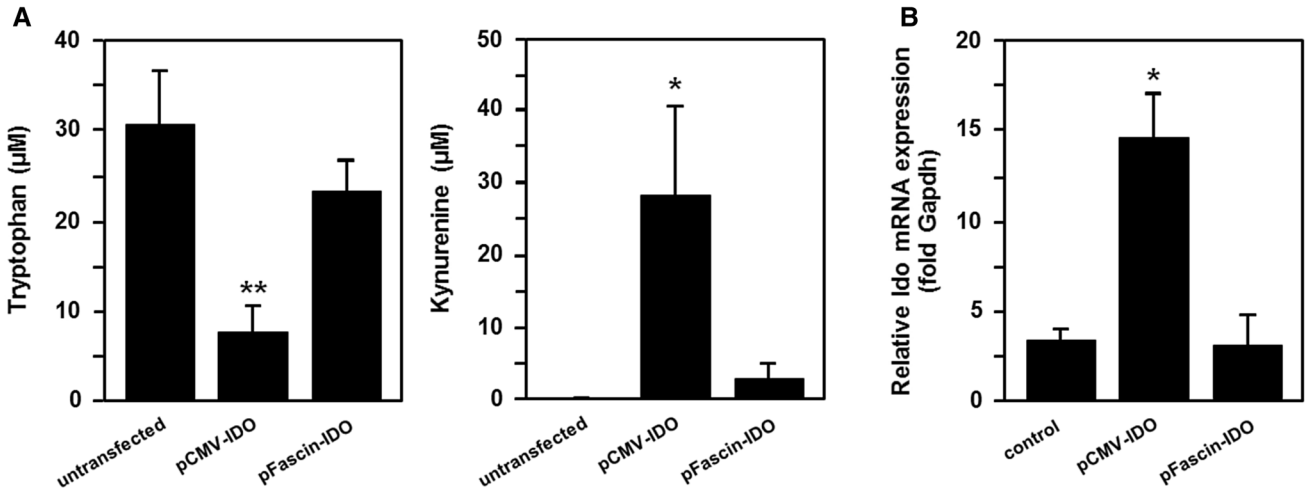

Fig. 1 Functional analysis of pCMV-IDO and pFascin-IDO in vitro and in vivo. **a** NIH-3T3 cells were transfected with pCMV-IDO or pFascin-IDO, each in combination with pCMV-EGFP to visualize transfection efficiency by fluorescence microscopy, using Fugene HD, or were mock transfected. After 1 day, medium was exchanged by culture medium containing Trp at defined concentration (25 µM). 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 ± 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$^+$ DCs were isolated by immunomagnetic sorting. IDO mRNA expression in CD11c$^+$ 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 ± 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 βGal-specific immune response by co-application of IDO-encoding plasmids

The βGal- 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-βGal, presentation of processed βGal-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-βGal 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 β-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 β-Gal-specific T-cell responses, where βGal expression was restricted to keratinocytes, was mediated by the transfer of βGal antigen to cutaneous DCs. Thus, it is plausible that the same mechanism of antigen transfer occurs in case of vaccination with CMV-β-Gal due to the ubiquitous activity of the CMV promoter (Ross et al. 2003). Keratinocyte-derived microvesicles containing β-Gal are then internalized by adjacent (non-transfected) cutaneous DCs present in the epidermis. Since PMED activates DCs, presentation of βGal-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 βGal-specific immune response. For this, mice were immunized three times in weekly intervals by PMED using either pFascin-βGal or pCMV-βGal, 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 βGal-specific IgG1 and IgG2 were determined by ELISA. As reported before (Sudowe et al. 2006), immunization of mice with pFascin-βGal + control plasmid yielded lower levels of β-Gal-specific IgG1 and IgG2a antibodies than immunization with pCMV-βGal + control vector (Fig. 2a, left panel). Furthermore, pCMV-βGal + control-vaccinated mice displayed higher titers of IgG1 than IgG2a, whilst the reverse was observed for mice transfected with pFascin-βGal (Fig. 2a, left panel). This finding is in accordance with our previous observations that antigen expression under the control of the Fascin-1 promotor 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 promotor 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-β (Castor et al. 2018). Similarly, co-application of pFascin-βGal and pCMV-IDO resulted in significantly reduced serum titers of both Ig isotypes as compared with control mice treated with pFascin-βGal + 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-βGal. When pCMV-βGal was used for immunization, neither co-application of pCMV-IDO nor pFascin-IDO inhibited Ig production.

In vitro, antigen-stimulated IFN-γ production was detected in culture supernatants of lymph node cells recovered from mice 13 days after the last immunization with pFascin-βGal and pCMV-βGal, 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-γ production, rather than a Th2-response, as indicated by the lack of IL-5. In pFascin-βGal-treated mice, co-application of pCMV-IDO, but not of pFascin-IDO, completely inhibited IFN-γ production (Fig. 2b, right panel). In the pCMV-βGal-treated mice, co-administration of pCMV-IDO did not exert an inhibitory effect on IFN-γ production (Fig. 2b, right panel).

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

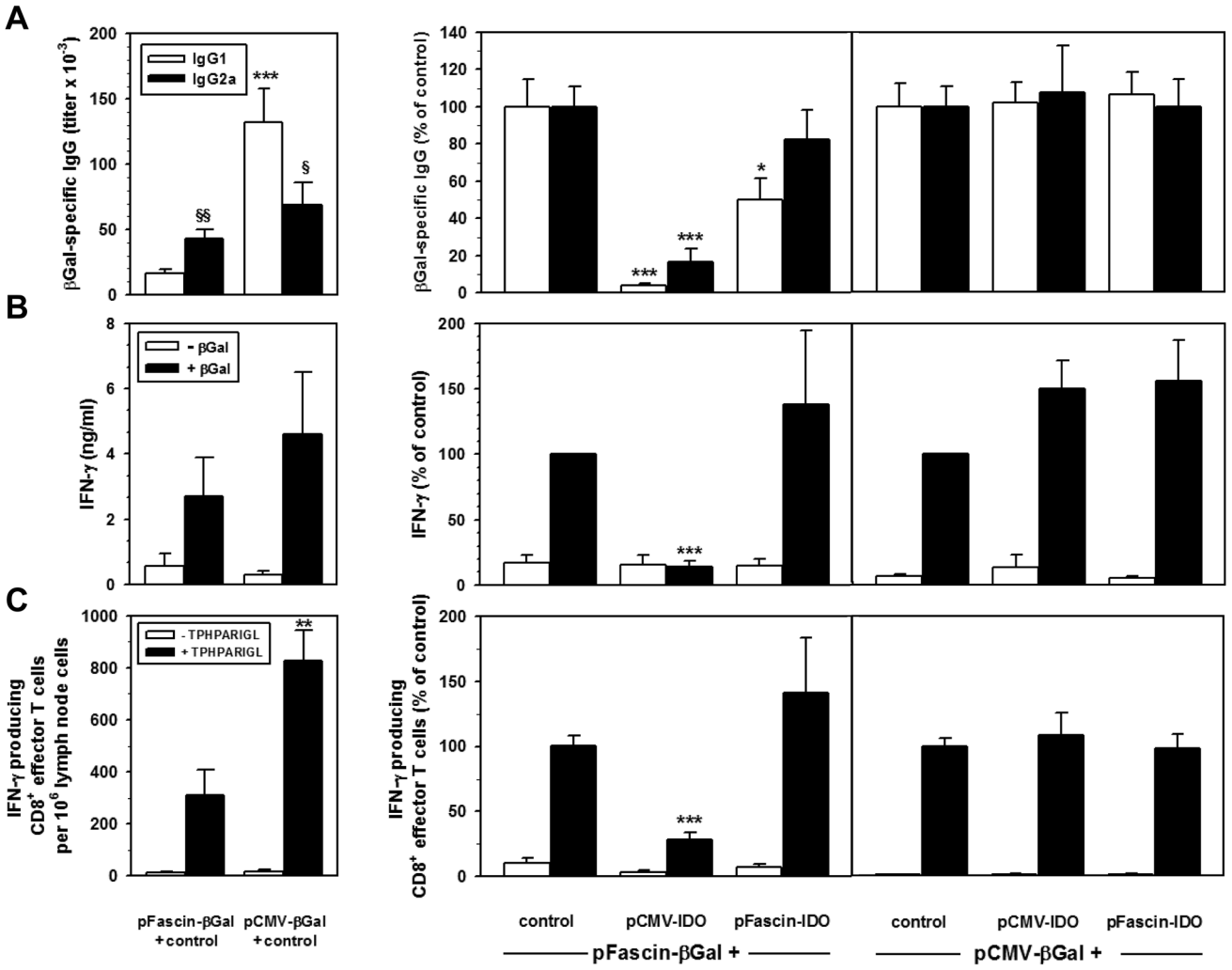

Fig. 2 Co-application of pFascin-βGal and pCMV-IDO strongly attenuates βGal-specific Th1/Th2/Tc1 responses. BALB/c mice (4 mice each/group and experiment) were immunized three times in weekly intervals by gene gun-mediated transfection of pFascin-βGal or pCMV-βGal, each in combination with control vector (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 $CD8^+$ 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-β-Gal) transgene expression (pCMV-βGal) tolerance may be induced. This requires ubiquitous co-expression of IDO (pCMV-IDO) to create a tolerance-promoting micromilieu. 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 pFscn-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 βGal-induced non-eosinophilic airway inflammation by biolistic co-application of βGal- 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 βGal-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 βGal protein on three consecutive days. Mice immunized with pFascin-βGal in combination with the control EGFP-encoding vector and subsequently provoked with βGal 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-βGal-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 βGal-challenged mice after PMED with pFascin-βGal (Fig. 3b, upper panel) and pCMV-βGal (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-βGal-treated mice. However, the reduction in neutrophil numbers in the group of pCMV-βGal-immunized mice, and IDO-mediated reduction in the numbers of lymphocytes in pFascin- and CMV-βGal-immunized mice were also not statistically significant.

These results show that transfection-mediated ubiquitous expression of IDO only in context with DC-restricted (pFascin-βGal) βGal 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 βGal-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-βGal for immunization, because pCMV-IDO did not exert beneficial effects, when co-applied with pCMV-βGal.

DC-focused biolistic co-application of βGal- and IDO-encoding plasmids does not affect the frequencies of Foxp3$^+$ regulatory T cells

IDO has been reported to exert its pro-tolerogenic effects via induction of CD4$^+$CD25$^+$Foxp3$^+$ Tregs (Mellor et al. 2017; Wu et al. 2018). So far, most studies have focused on the induction and functional properties of CD4$^+$ Treg (Klein et al. 2019). However, an increasing body of evidence has shown that CD8$^+$ T cells can differentiate to Foxp3$^+$ Treg as well (Yu et al. 2018). To assess the possibility that vaccination with CMV-IDO induced Foxp3$^+$ Treg, mice were vaccinated three times with pFascin-βGal 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$^+$ Tregs in lymphoid organs were determined by flow cytometry. All three groups of mice had comparable frequencies of CD4$^+$ and CD8$^+$ Foxp3$^+$ Tregs in lymph nodes (Supplemental Fig. 2, left panel) and spleen (Supplemental Fig. 2, right panel). However, since the determination of Foxp3$^+$ frequency was performed antigen-unspecifically on the total T-cell populations, we cannot rule out subtle alterations in the frequencies of β-Gal-specific Tregs. This result is reminiscent of our previous finding of a failure to induce Foxp3$^+$ Tregs by biolistic vaccination of mice by PMED with a MOG-encoding vector in combination with plasmids encoding IL-10 or TGF-β1 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-β, respectively (Ligocki and Niederkorn 2015). According to real-time PCR analysis, neither of these

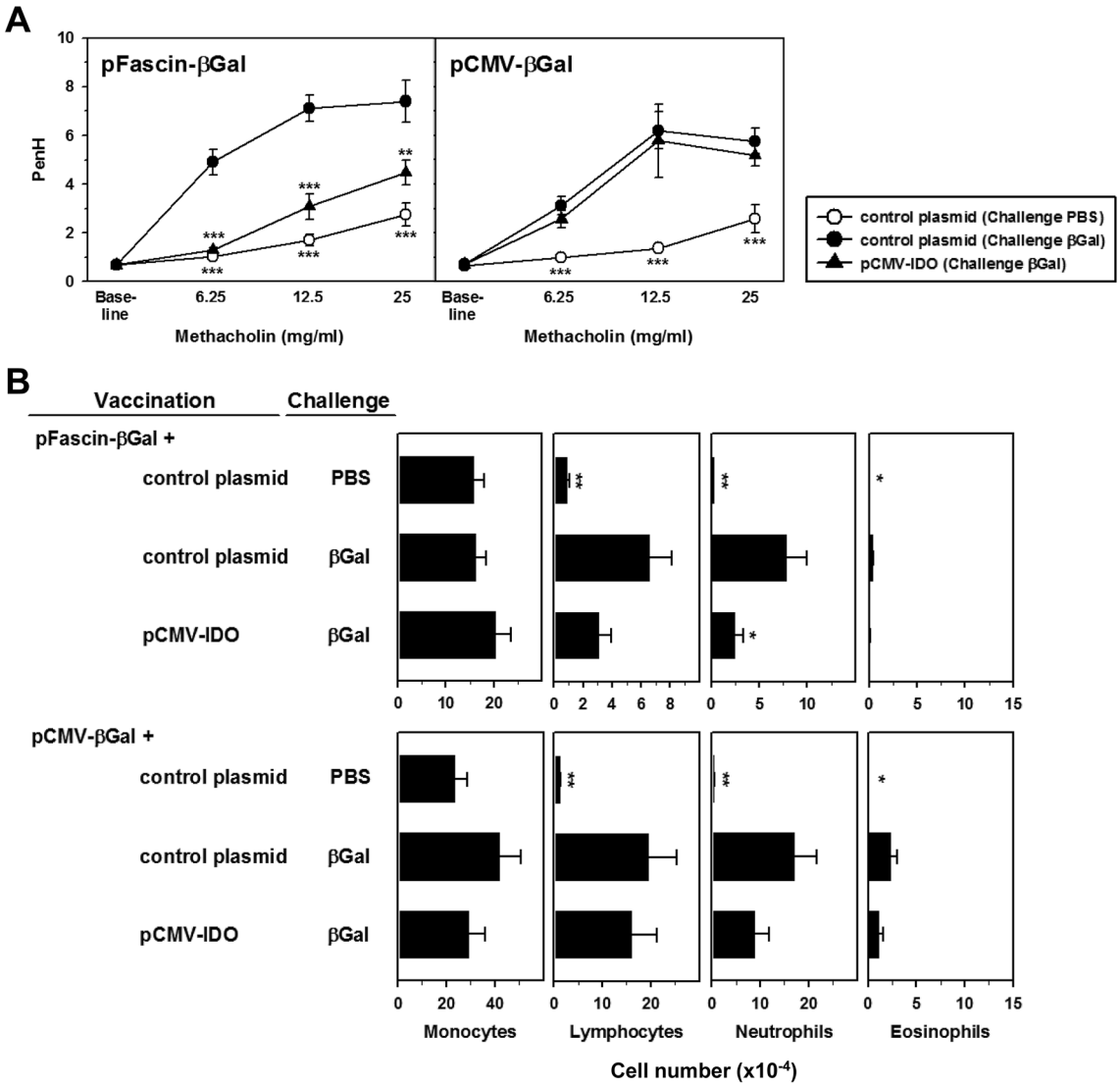

Fig. 3 Co-administration of pFascin-βGal and pCMV-IDO inhibits antigen-specific airway hyperresponsiveness. BALB/c mice were immunized three times in weekly intervals by gene gun-mediated transfection of pFascin-βGal or pCMV-βGal, each in combination with pCMV-IDO and control plasmid, respectively (see 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 5×10^5) were cytospun, stained with DiffQuick, and quantitated. **a**, **b** Data denote the mean ± 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 $CD4^+$ and $CD8^+$ 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-focussed biolistic co-application of βGal- and IDO-encoding plasmids in a mouse model of allergic airway disease

Next we asked whether PMED with βGal- and IDO-encoding vectors might exert an inhibitory effect on eosinophilic asthma induced by subsequent sensitization/provocation with βGal 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 βGal protein (prophylactic vaccination). Eight days after the last sensitization, mice were intranasally challenged with βGal protein on three consecutive days. Control mice, which did not receive PMED, but were sensitized with βGal protein and challenged with βGal protein developed a pronounced AHR as compared with mice challenged intranasally with PBS (Fig. 4a). PMED using pFascin-βGal 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 βGal 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 βGal-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-βGal + control plasmid, we observed a significantly reduced influx of eosinophils, which was not seen with pFascin-βGal + pCMV-IDO-vaccinated mice.

Our results are in line with earlier findings showing that prophylactic vaccination with pFascin-βGal augmented lung

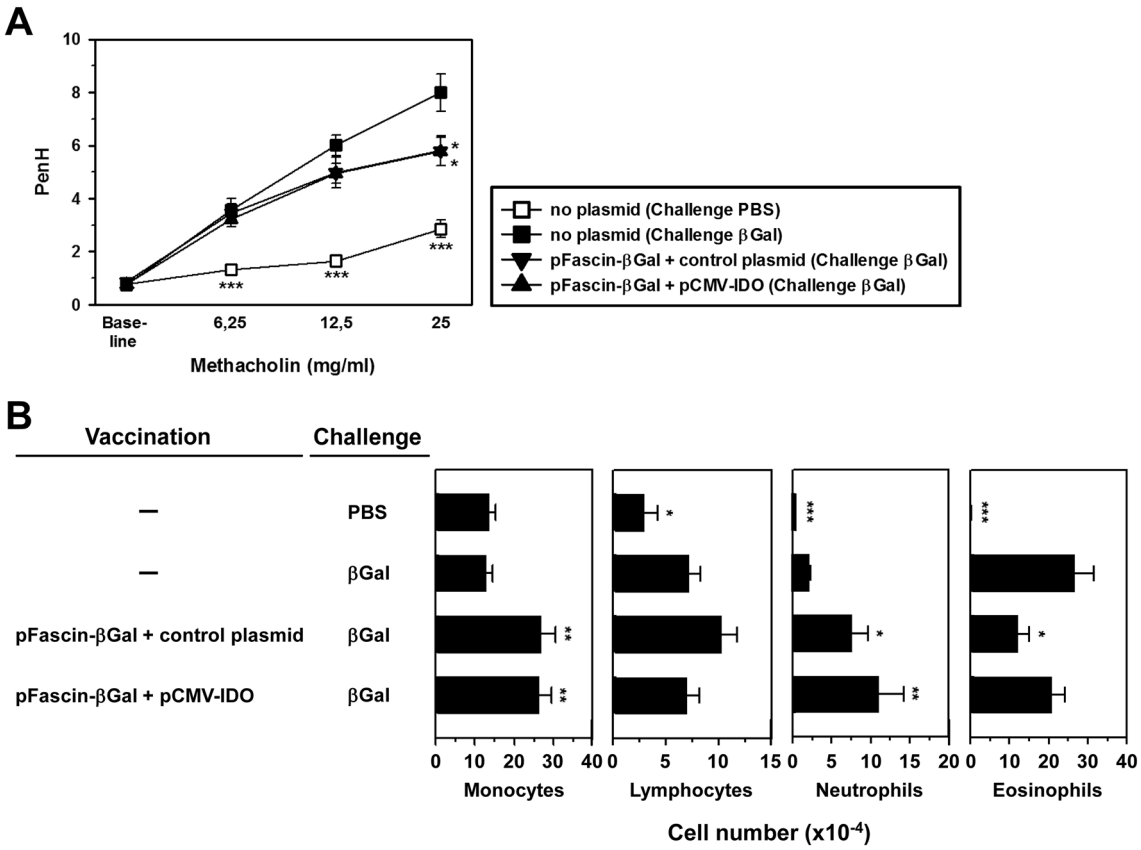

Fig. 4 Prophylactic vaccination with pFascin-βGal and pCMV-IDO does not affect antigen-induced AHR. BALB/c mice (each 4 mice/group and experiment) were immunized three times in weekly intervals by gene gun-mediated transfection of pFascin-βGal in combination with the control plasmid or pCMV-IDO, respectively, or were left untreated. One week after the last immunization, mice were sensitized three times in weekly intervals by s.c. injection of βGal protein (10 µg). After 8 days, mice were intranasally provoked with βGal-protein on three consecutive days. One group of control mice was treated with PBS. **a** One day after the last application of βGal 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 ± SEM of 16 mice per group compiled from four independent experiments. Statistically significant differences versus the non-vaccinated control group challenged with βGal 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 βGal-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 βGal 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 βGal protein. This induced a pronounced type 2 immune response as verified by high-βGal-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-βGal together with pCMV-IDO and control plasmid, respectively. The remaining two groups were left untreated. When the latter two groups were intranasally challenged with βGal and PBS, respectively, we observed pronounced AHR after the application of βGal in contrast to treatment with PBS (Fig. 5a). After challenge of the two vaccinated groups of mice with βGal, 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

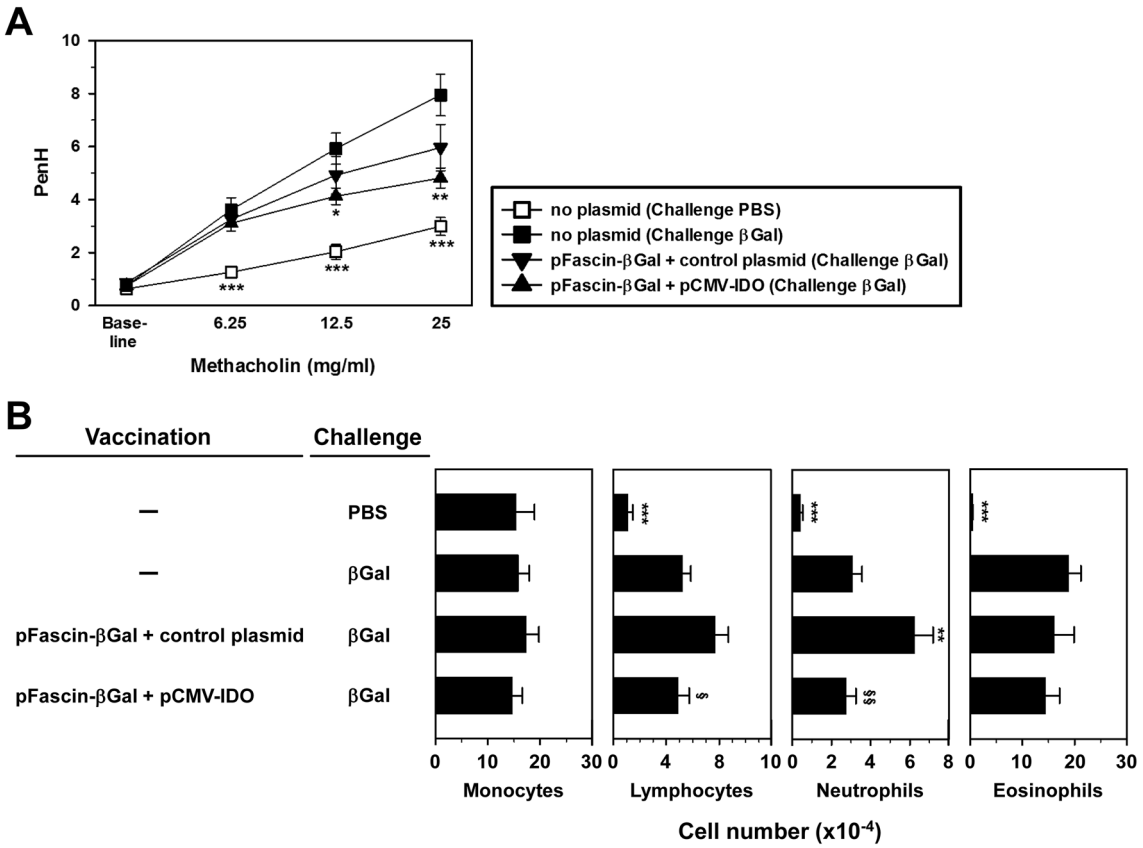

Fig. 5 Therapeutic vaccination with pFascin-βGal and pCMV-IDO attenuates AHR. BALB/c mice (each 4 mice/group and experiment) were sensitized three times in weekly intervals by s.c. injection of βGal or were left untreated. Five days after the last sensitization, titers of βGal-specific antibodies were 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 biolistically transfected three times in weekly intervals with pFascin-βGal + control plasmid and pFascin-βGal + pCMV-IDO, respectively. Two weeks later, all mice were intranasally provoked with βGal protein on three consecutive days. **a** One day after the last application of βGal 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 ± 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 βGal 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 βGal versus PBS (Fig. 5b). In mice vaccinated with pFascin-βGal 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 β-Gal protein. In contrast, co-treatment of mice with pFascin-βGal 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, $CD4^+$ and $CD8^+$ 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 βGal 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 micromilieu 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 $CD4^+$ Th1 cells (Xu et al. 2008) and $CD8^+$ 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 βGal 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.

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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.

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Inhibition of antigen-specific immune responses by co-expression of an indoleamin2,3-dioxygenase (IDO)-encoding vector requires antigen transgene expression focused to dendritic cells

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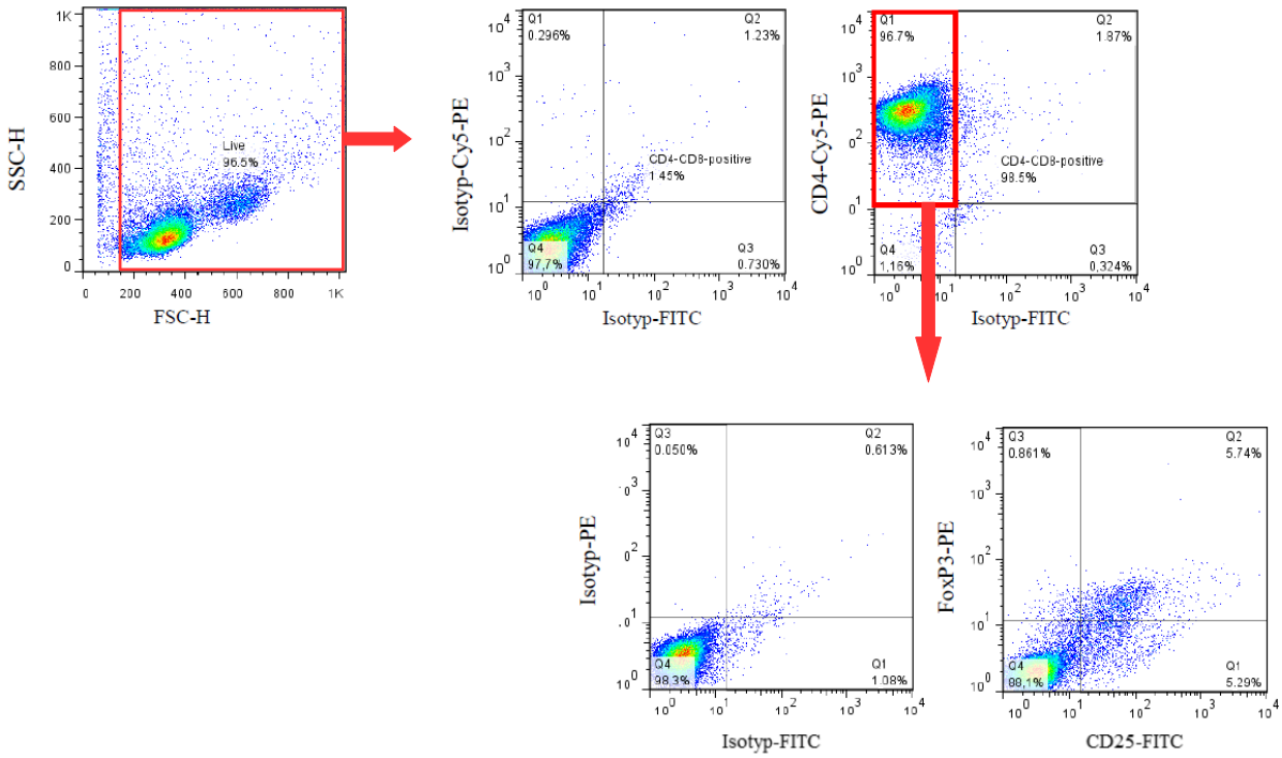
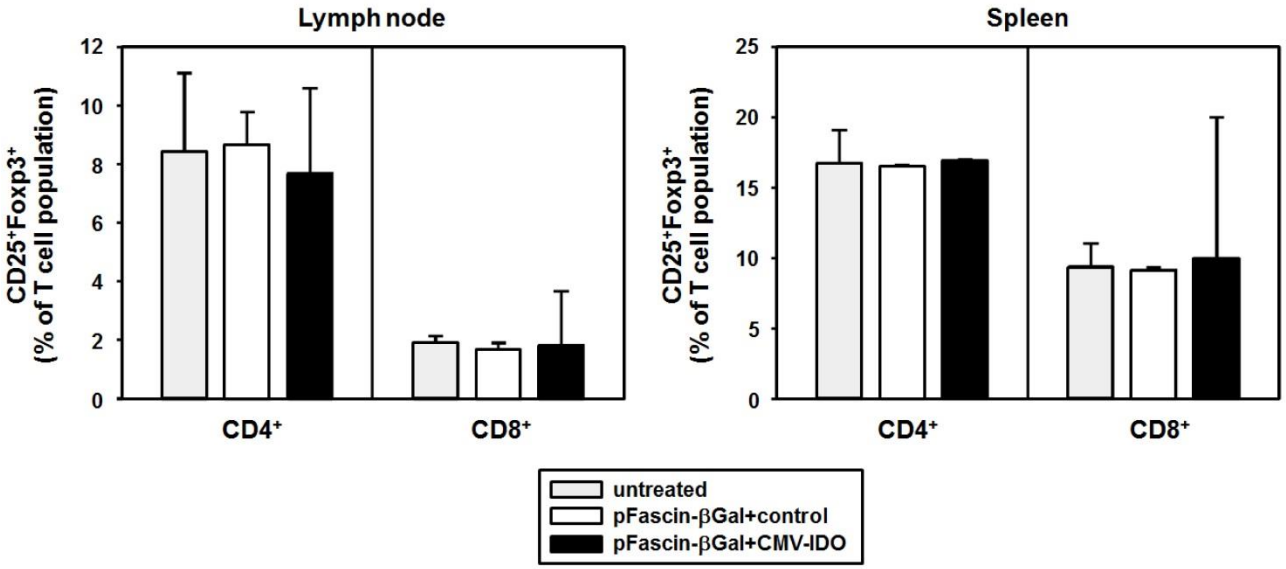
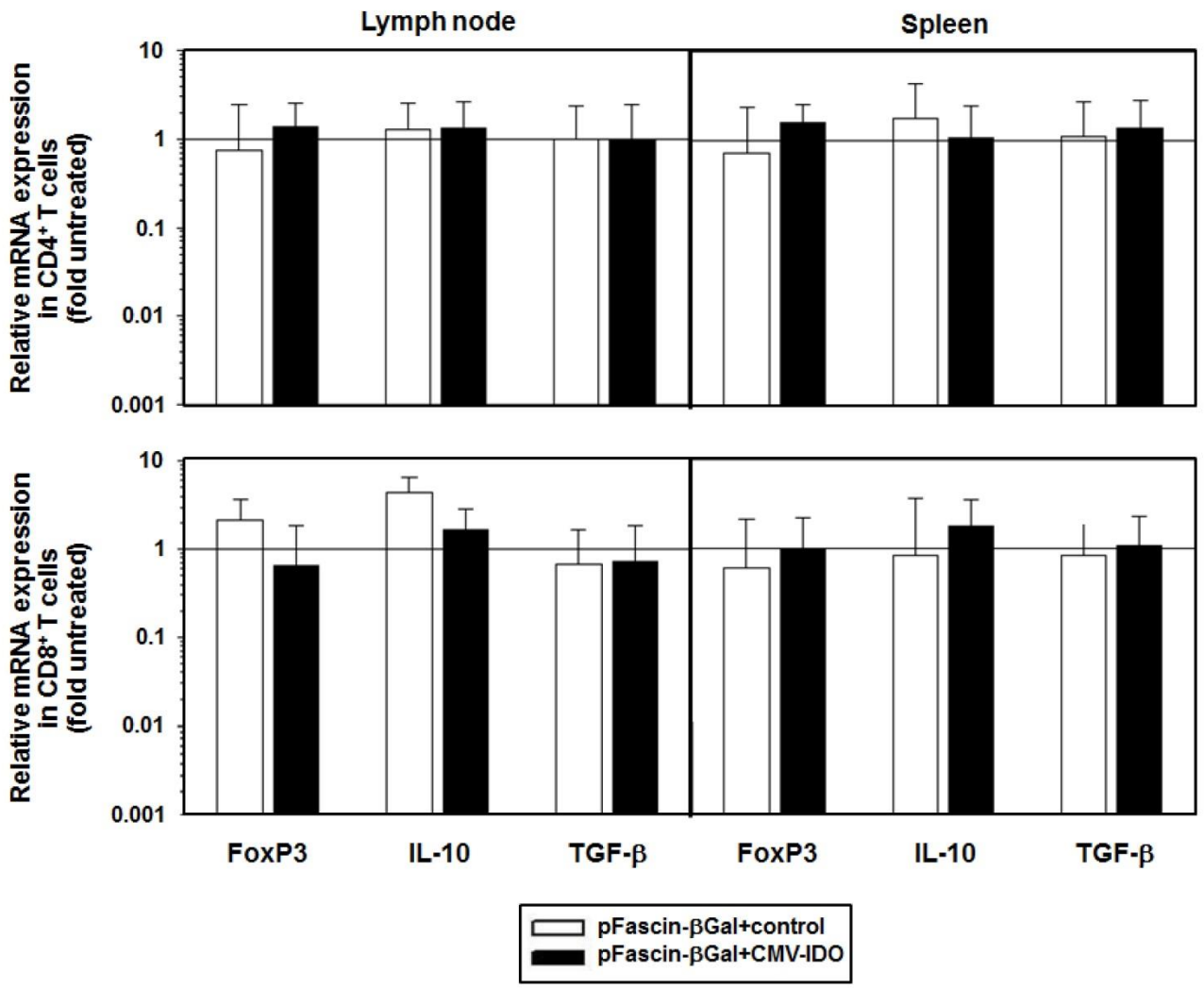


Supplermental Fig. 1 Gating strategy for assessment of $CD4^{+}CD25^{+}Foxp3^{+}$ regulatory T cells.



Supplemental Fig. 2 IDO transgene expression does not alter the frequency of Foxp3$^+$ regulatory T cells. BALB/c mice (each 3 mice/group and experiment) were immunized three times in weekly intervals by gene gun-mediated transfection of pFascin-ßGal in combination with the control plasmid or pCMV-IDO, respectively, or were left untreated (see legend of Fig. 2). One week later, axillary and inguinal lymph nodes and spleens were retrieved, and T cell populations (CD4$^+$, CD8$^+$) were immunomagnetically isolated from pooled lymph node and spleen cell populations, respectively. Frequencies of T cell populations that coexpressed CD25 and Foxp3 were assayed by flow cytometry (the gating strategy is shown in Supplemental Fig. 1). Data denote the mean±SEM of a total of each 6 mice compiled from two independent experiments.



Supplemental Fig. 3 IDO transgene expression has no effect on T cell-specific expression of antiinflammatory cytokines. BALB/c mice were immunized, and T cell populations were isolated from lymph nodes and spleen as described in the legend of Fig. 4. Expression of mRNA species encoding Foxp3, IL-10, TGF-ß were assessed by real-time PCR. Data denote relative expression of the candidate mRNA species normalized to expression of the house-keeping gene GAPDH in each group, and are given as fold of expression in the according T cell population isolated from untreated mice. Data denote the mean±SEM of a total of 6 mice compiled from two independent experiments.