

LIPOPOLYSACCHARIDE STIMULATION OF DENDRITIC CELLS INDUCES INTERLEUKIN-10 PRODUCING ALLERGEN-SPECIFIC T CELLS IN VITRO BUT FAILS TO PREVENT ALLERGIC AIRWAY DISEASE

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□ *Dendritic cells (DCs) play an important role in directing naïve T cells towards a Th1/Th2 or regulatory T cells (Treg) cell phenotype. In this context, interleukin (IL)-10 has been shown to exhibit immune regulatory capacities. The aim of this study was to delineate the influence of high-IL-10-producing DCs on DC-T-cell interactions in inhibiting allergen-induced airway inflammation and hyperreactivity in a murine model of allergic airway disease. Bone marrow-derived dendritic cells (BMDCs) were generated from hemopoietic progenitors by culture with granulocyte-macrophage colony-stimulating factor (GM-CSF), and stimulated with ovalbumin (OVA) ± lipopolysaccharide (LPS). The effects of ovalbumin-pulsed BMDCs on cytokine production by allergen-specific naïve T cells were studied in vitro. The development of airway inflammation in Balb/c mice was determined after intranasal administration of BMDCs in vivo. LPS stimulation of BMDCs strongly enhanced IL-10 production. Coculture of LPS-modulated DCs exhibiting increased IL-10 production with allergen-specific naïve T cells reduced the production of interferon (IFN)- γ and IL-5, but enhanced the production of IL-10. After blockade with anti-IL-10 plus anti-IL-10-receptor antibodies, the level*

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of IFN- γ and IL-5 production by cocultured T cells was restored, underlining the regulatory function of IL-10. Intranasal administration of high-IL-10-producing LPS-stimulated, OVA-primed BMDCs prior to repetitive airway allergen challenges resulted in an even enhanced airway inflammation. These data demonstrate that increased IL-10 production by DCs may be a critical element for T-cell activation and differentiation in the context of allergen-induced immune responses in vitro. However, this DC modulation did not translate into suppression of allergic airway disease in vivo.

Keywords allergic airway disease, dendritic cells, IL-10

T-helper 1 and T-helper 2 T cells (Th1, Th2) represent terminally differentiated effector T cells. They orchestrate cellular or humoral immune defense mechanisms that involve the expression of distinct cytokine patterns. Yet little is known about the exact mechanisms that control the initial T-cell polarization [1]. For this initial priming of naïve T cells, the interaction with antigen-presenting cells (APCs) or dendritic cells (DCs) is crucial. These APCs become activated primarily by certain stimuli such as characteristic pathogen-associated molecular patterns (PAMPs) or proinflammatory cytokines. The ensuing maturation of DCs results in enhancing T-cell stimulation via costimulatory signals and cytokine production by the DCs.

For Th2-biased allergic diseases such as asthma, DCs are thought to be responsible for the capturing of allergens in the airways and the shuttling of processed allergens to the draining lymph nodes where antigen (Ag) presentation and T-cell activation take place.

A critical cytokine produced by DCs involved in Th polarization is interleukin (IL)-12, which is a major factor for Th1 differentiation [2–4]. Accordingly, low IL-12 levels in activated DCs are associated with the risk of developing Th2 immune response to allergens, whereas high levels of IL-12 seem to offer protection from allergic diseases. Stimuli that are known to induce high IL-12 production by DCs are, among others, cell membrane components from gram-negative bacteria such as lipopolysaccharides (LPS). Indeed, the production of IL-12, as well as IL-1 β , tumor necrosis factor (TNF)- α , and IL-10 by bone marrow-derived dendritic cells (BMDCs) has been reported [5, 6].

Another DC cytokine that can affect T-cell polarization is IL-10. IL-10 is often coinduced with IL-12 in DCs following bacterial stimulation. This has led to the hypothesis that IL-10 might serve as a counter signal for blocking exaggerated T-cell responses induced by IL-12 [7]. Recent studies have shown that exogenous IL-10 inhibits LPS- and CD40-induced IL-12 production by DCs and thus impairs their ability to induce Th1 cells in vivo [8, 9]. On the other hand, the addition of IL-10 neutralizing antibody or soluble IL-10 receptor (IL-10R) to DCs has resulted in opposite effects [10]. This property of IL-10 points towards a more regulatory role resulting in the induction of allergen specific tolerance [11,12].

The aim of the present study was to delineate whether in vitro induction of high IL-10 production by DCs translates to a regulatory function on T-cell differentiation and thus suppressed T cell-dependent immune responses in vivo.

MATERIAL AND METHODS

Animals

All experiments were approved by the local ethics committee. Specific pathogen-free female BALB/c mice (20 to 23 g) and OVA-TCR transgenic DO11.10 mice were bred in the animal facility of the Federal Institute for Risk Assessment, Berlin. Mice were maintained on ovalbumin (OVA)-free diet and kept in isolated ventilation cage systems.

Generation of Bone Marrow-Derived Dendritic Cells

Bone marrow-derived dendritic cells were generated from bone marrow of BALB/c mice in the presence of high dosed granulocyte-macrophage colony-stimulating factor (GM-CSF), as previously described [13]. Briefly, freshly prepared bone marrow cells were cultured in petridishes (BD Labware, Le pont de Claix, France) with RPMI 1640 medium (Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated fetal calf serum (PAA Laboratories, Linz, Austria), 2 mM L-glutamine, 10 mM HEPES buffer, 60 $\mu\text{g}/\text{mL}$ penicillin, and 20 $\mu\text{g}/\text{mL}$ gentamicin in the presence of 10 ng/mL GM-CSF (PeproTech). After 10 days, nonadherent cells were counted, resuspended at 1×10^6 cells/mL, and stimulated in 24-well plates (NUNC, Neerijse, Belgium) for 18 hours with 100 $\mu\text{g}/\text{mL}$ OVA (Sigma) alone or in combination with 10 or 100 ng/mL LPS (Sigma, St. Louis, USA). BMDCs resuspended only with supplemented RPMI medium were used as controls. The culture supernatants were harvested and tested for the presence of cytokines by enzyme-linked immunosorbent assay (ELISA), whereas cells were harvested for phenotypical characterization by flow cytometry after exclusion of dead cells by propidium iodide (PI) staining (data not shown).

Analysis of OVA by *Limulus* amebocyte lysate (LAL) test revealed quantities of LPS ranging between 6 and 60 ng/mg endotoxin.

In Vitro Stimulation of Naïve OVA-Specific CD4⁺ T Cells

Spleen cells from DO11.10 mice were prepared and CD4-enriched by magnetic cell sorting (MACS) with the CD4 microbead L3T4 (Miltenyi Biotech, Bergisch-Gladbach, Germany) after CD11c depletion by using a LS separation column (Miltenyi Biotech). OVA (100 $\mu\text{g}/\text{mL}$) or OVA

(100 $\mu\text{g/mL}$)/LPS (10 ng/mL)–stimulated BMDCs were harvested and washed and cocultured with a DC:CD4 ratio of 1:10 at 10^6 cells/mL using 24-well plates (NUNC). Unstimulated BMDCs were cocultured as negative control. To determine the effect of IL-10, 1 $\mu\text{g/mL}$ anti-mouse IL-10R antibody (1B1.3a; Pharmingen) and 10 $\mu\text{g/mL}$ anti-mouse IL-10 antibody (JES5-2A5; Pharmingen) were added during coculture. After 6 days of coculture, cells were harvested, washed, and restimulated with phorbol myristate acetate (PMA) (10 ng/mL ; Sigma-Aldrich) and ionomycin (1 $\mu\text{g/mL}$; Sigma-Aldrich) for 24 hours. IL-5, IL-13, IL-10, and interferon (IFN)- γ levels in supernatants were detected by ELISA and cells were further characterized by flow cytometry.

BMDC Application and Antigen Challenge In Vivo

Naïve female BALB/c mice were slightly anaesthetized with ketamine i.p. BMDCs stimulated with OVA (100 $\mu\text{g/mL}$) and LPS (100 ng/mL) or OVA (100 $\mu\text{g/mL}$) alone were administered intranasally in a concentration of 10^6 cells suspended in 100 μL phosphate-buffered saline (PBS) on day 0. Unstimulated BMDCs were used as a negative control. Ten days after DC transfer, animals were challenged with 50 μg OVA intranasally (i.n.) on 4 consecutive days. Shorter (less than 6 days) period between allergen airway challenges and DC transfer did not result in significant induction of allergic immune or airway responses (data not shown). On day 15, airway reactivity was determined. For further analysis, mice were sacrificed 24 hours later.

Determination of Airway Reactivity

Airway reactivity (AR) was determined by barometric plethysmography (Buxco), as previously described [14, 15]. Mice were challenged with aerosolized PBS or increasing doses of methacholine (MCh; 6 to 50 mg/mL) for 3 minutes. Data are expressed as fold increase of baseline values with PBS, using the dimensionless parameter “enhanced pause” (P_{enh}).

Preparation of Bronchoalveolar Fluid

On day 16, the animals were sacrificed and tracheas cannulated. Airways were lavaged twice with 0.8 mL ice-cold PBS, and cell numbers and cytokine contents were determined as described previously [14, 16]. Bronchoalveolar (BAL) cells were counted, spun onto glass slides using a cytospin centrifuge (Shandon Southern Products, Asmoor, UK), and stained with Diff-Quick (Dade Behring, Marburg, Germany) according to the manufacturer’s instructions. Numbers of eosinophils, neutrophils, lymphocytes, and macrophages present in the BAL were determined microscopically using standard histological criteria. Cell-free bronchoalveolar lavage fluid (BALF) was frozen at -20°C until further analysis.

Assessment of Immunoglobulins

Total immunoglobulin E (IgE) and allergen-specific IgE, IgG1, and IgG2a antibody titers were measured by enzyme-linked immunosorbent assay (ELISA), as described previously [14].

Enzyme-Linked Immunosorbent Assay

Cytokine profiles were analysed using ELISA kits for IL-10, IL-12p70, tumor necrosis factor (TNF)- α , and IFN- γ (OptEIA, BD Biosciences, San Diego, USA) according to the manufacturer's instructions. To determine cytokine levels of IL-5 and IL-13, established ELISA protocols (BD Biosciences) were performed.

Histochemistry

Animals were sacrificed, and lungs were removed and shock frozen in liquid nitrogen. Ten-micrometer sections were cut on a cryostat, mounted on glass slides, and rinsed with PBS.

Staining with hematoxylin and eosin (H&E) was carried out using standard protocols.

Statistical Analysis

Results are presented as mean values $\pm$ standard deviation. Means were compared by using Mann-Whitney *U* test, SPSS, version 7.5.2G (SPSS, Chicago, IL). *P* values $< .05$ were regarded as significant differences. Each experiment was performed in a minimum of 3 independent setups.

RESULTS

LPS and OVA Induce Phenotypic and Functional DC Maturation and IL-10 Production

Immature bone marrow-derived DCs were exposed to LPS and OVA and phenotypical and functional markers of DC maturation were analyzed (supplementary material, Figure S1). Exposure of immature DCs to OVA alone induced an up-regulation of C40, CD80, and CD86, which was even more enhanced after additional stimulation with LPS.

DCs did not spontaneously secrete detectable levels of IL-10 (<4 pg/mL), IL-12p70 (<16 pg/mL), and TNF- α (<4 pg/mL) (Figure 1). OVA stimulation did not significantly induce secretion of these cytokines either. A simultaneous stimulation with OVA and LPS led to a 3-times higher IL-10 production, compared to stimulation with OVA alone. The production of TNF- α , IL-12p70, and IL-1 β after LPS stimulation was strongly

Cytokine production by BMDC after stimulation +/- LPS

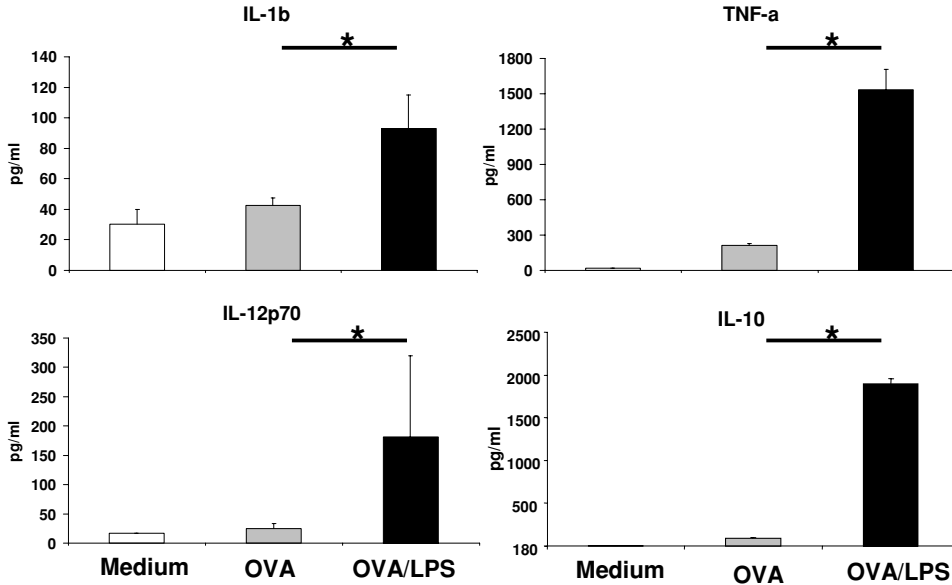


FIGURE 1 Cytokine profile of stimulated BMDCs. After 10 days of culture, BMDCs were stimulated with 100 $\mu\text{g}/\text{ml}$ OVA (OVA) either alone or together with 100 ng/mL LPS (OVA/LPS). Eighteen hours later, supernatants were collected and taken for cytokine analysis by ELISA. Secretion levels of IL-10, IL-12p70, IL-1 β , and TNF- α are shown as mean $\pm$ SD of triplicate samples.

up-regulated as well, compared to stimulation with OVA alone. These data show that the amount of LPS in OVA (LAL test 6 to 60 ng/mg endotoxin; stimulation with 100 $\mu\text{g}/\text{mL}$ OVA) is not sufficient enough to induce appreciable levels of cytokines. However by adding 20 to 200 times more LPS (100 ng/mL) exogenously, the cytokine production by DCs was strongly up-regulated.

Compared to stimulation with OVA/LPS, we did not find significant differences in cytokine production by DCs stimulated only with LPS (see Figure S2, supplementary material). Addition of OVA was yet required in view of the coculture experiments with allergen-specific T cells *in vitro* and the experiments with allergen challenges *in vivo*.

LPS-Stimulated DCs Reduce Cytokine Production by CD4⁺ T Cells *In Vitro*, Which Is Restored After IL-10 Blockage

To determine whether BMDCs modulated through LPS act differently in directing naïve T-cell differentiation, we cocultured them with naïve CD4⁺ T

cells from syngenic OVA peptide-specific TCR transgenic mice (DO11.10). Following 6 days of coculture, cells were harvested, washed, and restimulated with PMA and ionomycin for 24 hours. At this time point, no BMDCs were left. The restimulated CD4⁺T cells cocultured with LPS and antigen-treated DCs produced lower levels of IL-5 and IFN- γ , in comparison to T cells stimulated by DCs stimulated with OVA alone. Notably, the IL-10 production of T cells was significantly enhanced after cocultivation with OVA + LPS-stimulated DCs (hereafter called high-IL-10-producing DCs, OVA/LPS-pulsed DCs), compared to DCs stimulated with OVA alone (OVA-pulsed DCs). To rule out IL-10 as an autocrine inhibitor, DCs were stimulated with LPS in the presence of neutralizing and blocking IL-10 antibodies (anti-mouse IL-10R and anti-mouse IL-10 antibody). Interestingly, IL-10 blockage and neutralization of DCs restored IL-5 and IFN- γ release (Figure 2).

These results suggest that LPS can modulate the function of DCs by suppressing the production of cytokines such as IL-5 as well as IFN- γ . IL-10, which is strongly expressed after LPS stimulation, has a suppressive function, because blockage and neutralization of IL-10 on DCs has a restorative effect on the cytokine production of cocultivated T cells.

Effects of DC Transfer on Allergen-Induced Airway Inflammation In Vivo

We continued by analyzing the effect of IL-10-producing BMDC in an in vivo model for allergic airway inflammation. Therefore, antigen-pulsed and -stimulated BMDCs or unstimulated BMDCs were administered intranasally to naïve BALB/c mice. After 10 days, animals were antigen challenged by intranasal application on 4 consecutive days. Animals of the group which received only OVA-pulsed BMDCs showed nearly 2.5 times higher total cell numbers in the BALF (examined 48 hours after the last challenge) compared to the control group, which received unpulsed BMDCs. Treatment with OVA-pulsed DCs caused allergen-induced airway inflammation, shown by significant influx of cells into the airways, mainly lymphocytes and eosinophils (Figure 3), demonstrating that the transfer of antigen-pulsed DCs drives the priming of naïve endogenous T cells in recall to allergen-specific activation in our system. However, the animals of the OVA/LPS group showed an even higher total cell number in the BALF compared with the control group receiving unpulsed DCs (approximately 3.5 times increase), although high-IL-10-producing BMDCs stimulated with LPS had been administered.

In the BALF, we detected a significant increase of IL-5 and IL-13 after OVA-pulsed DC application and an even stronger increase after administration of OVA/LPS-pulsed DCs. We could not detect relevant levels of IFN- γ , IL-4, and TNF- α (data not shown).

Cytokine-production of allergen-specific T-Cells co-cultured with pulsed BMDC

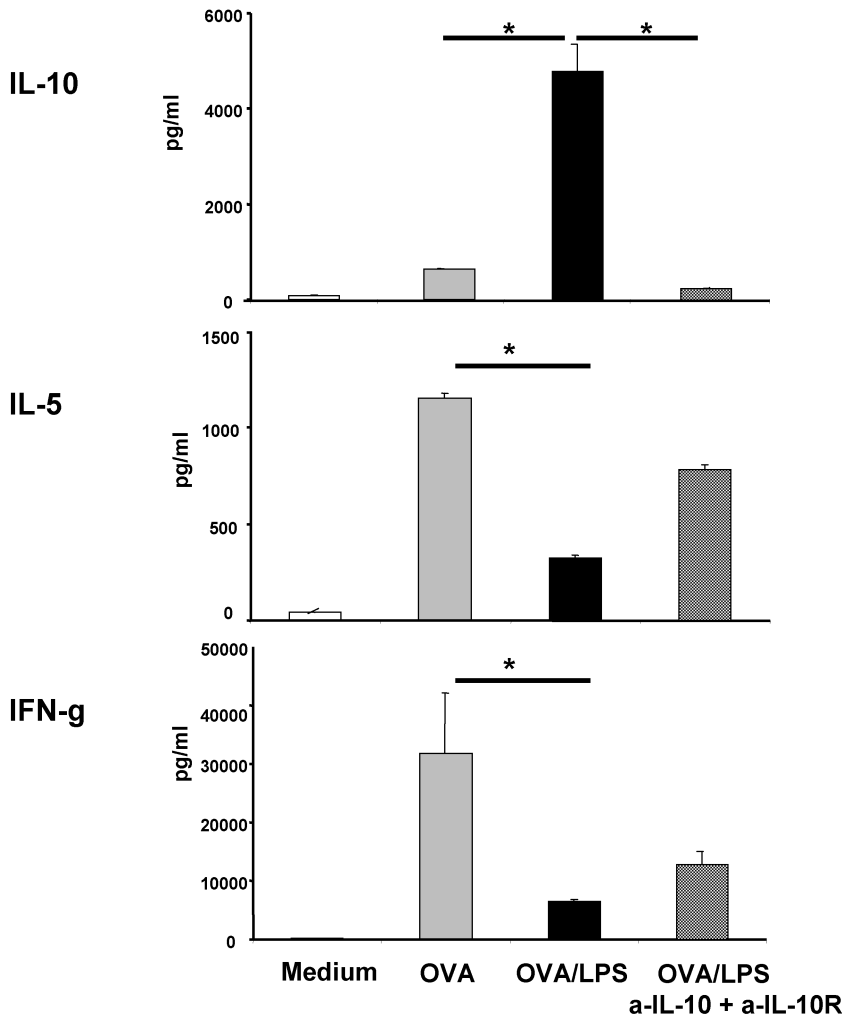


FIGURE 2 Cytokine profile of CD4⁺ T cells after coculture with stimulated BMDCs. BMDCs were pulsed with 100 μ g/mL OVA (OVA) and stimulated with 10 ng/mL LPS (OVA/LPS) before coculture with CD11c-depleted and CD4-enriched spleen cells from OVA-TCR transgenic DO11.10 mice. Unstimulated BMDCs were cocultures with CD4⁺ spleen cells as negative control (medium). After a 6-day coculture, cells were harvested and washed before restimulation with 10 ng/mL PMA and 1 μ g/mL ionomycin. Twenty-four hours later, supernatants were collected to determine cytokine levels by ELISA. Levels of cytokine secretion are shown for IL-10, IL-5, and IFN- γ as mean $\pm$ SD of triplicate samples.

Lung sections maintained from sacrificed mice after treatment protocol on day 16 showed enhanced peribronchial inflammation in both experimental groups, compared with control animals treated only with unpulsed BMDCs and allergen challenge. Animals showed increased peribronchial

***In vivo* transfer of BMDC: BAL**

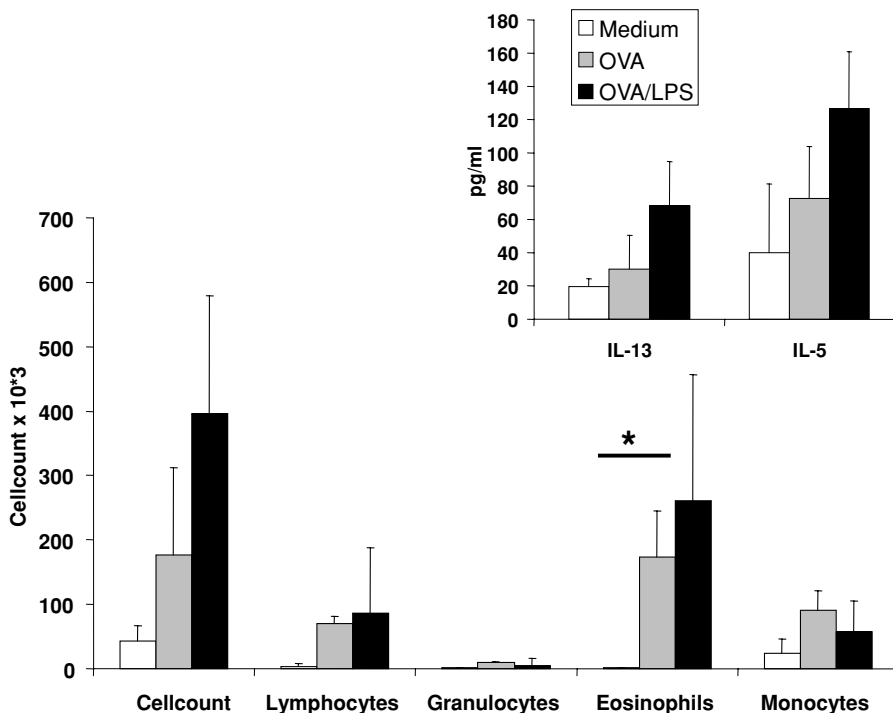


FIGURE 3 Cell count and differentiation in bronchoalveolar fluid. A total of 10^6 OVA ($100 \mu\text{g/mL}$)-pulsed BMDCs (OVA) or OVA-pulsed BMDCs stimulated with 100 ng/mL LPS (OVA/LPS) were administered i.n. in naïve BALB/c mice. Unstimulated BMDCs (Medium) were used as negative control. Starting on day 10, i.n. antigen challenge with $50 \mu\text{g}$ OVA was performed on 4 consecutive days. Forty-eight hours after the last challenge, animals were sacrificed and tracheas cannulated for BAL. Cells from BALF were counted, spun onto glass slides, and stained with DiffQuick before microscopy. Total cell number and numbers of different cell types are shown as mean $\pm$ SD of 5 animals per group in a representative assay from a series of three independent experiments. BAL fluids were also taken for cytokine analysis by ELISA. Secretion levels of IL-13 and IL-5 are shown as mean $\pm$ SD of triplicate samples.

infiltrates after the administration of LPS-stimulated and OVA-pulsed BMDCs (OVA/LPS group) in comparison to those treated with unstimulated OVA-pulsed BMDCs (Figure 4).

Effects of DC Transfer on Total and OVA-Specific Immunoglobulin Production In Vivo

The *in vivo* transfer of modulated DCs did not lead to different serum levels of total and specific IgE between the experimental groups. However, OVA-specific IgG1 was slightly enhanced after OVA-pulsed DC application, whereas OVA-specific IgG2a was increased significantly after application of OVA/LPS-pulsed DCs (Figure 5).

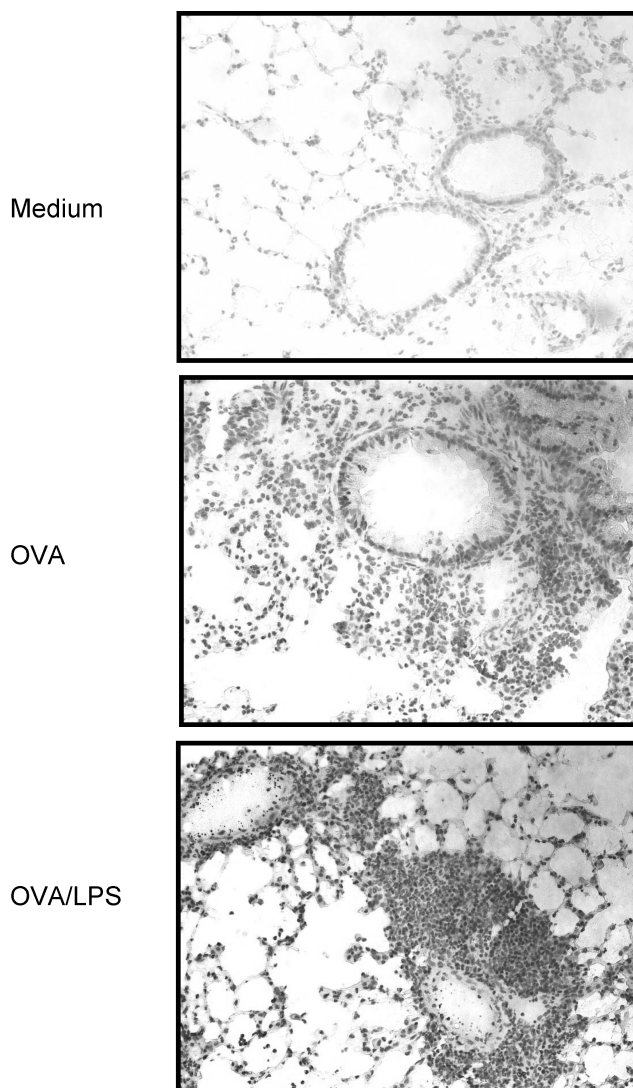


FIGURE 4 Light photomicrographs of H&E-stained cryosections of lung tissues. Sixteen days after i.n. administration of 10^6 BMDCs pulsed with 100 $\mu\text{g}/\text{mL}$ OVA (*middle panel*) or pulsed with 100 $\mu\text{g}/\text{mL}$ OVA and 100 ng/mL LPS (*bottom panel*) into naïve female BALB/c mice, the animals were sacrificed and lungs frozen with liquid nitrogen. Animals receiving unstimulated BMDCs were regarded as negative controls (*top panel*). Animals of all groups regain an i.n. allergen challenge with 50 μg OVA on 4 consecutive days ending 48 hours before analysis. Ten-micrometer cryosections from lung tissue were fixed and stained with H&E.

Determination of Airway Responsiveness After Application of Modulated DC In Vivo

In vivo airway responsiveness to aerosolized MCh in spontaneously breathing, conscious mice was assessed by barometric whole-body plethysmography [14, 15]. Following application of OVA-pulsed DCs in naïve mice,

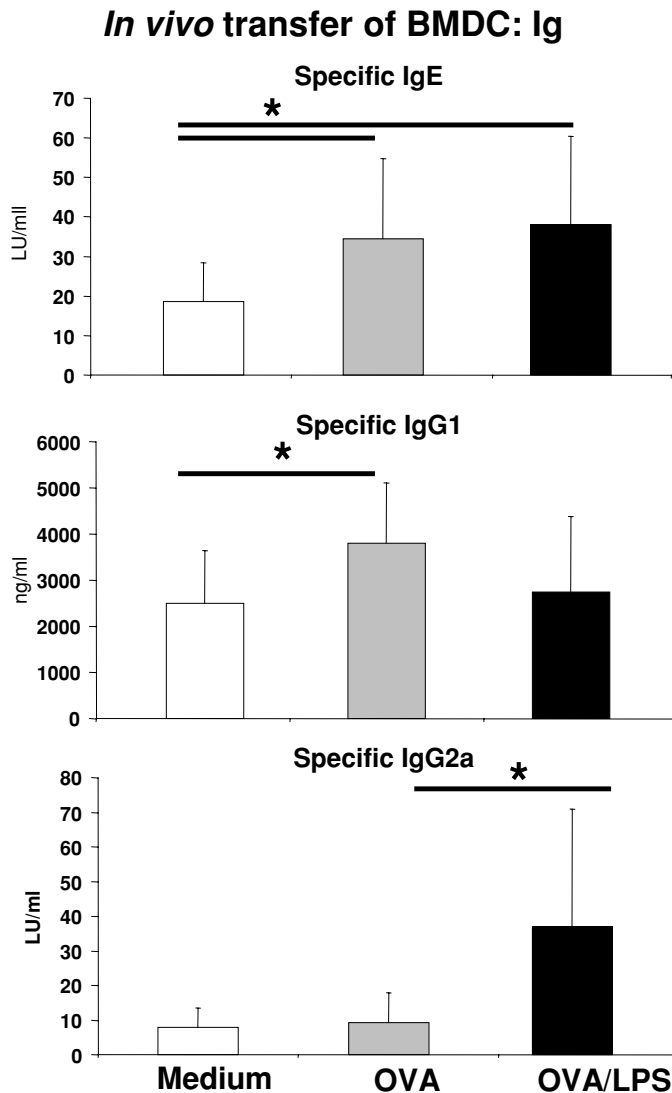


FIGURE 5 Immunoglobulin levels after *in vivo* transfer of stimulated BMDCs and intranasal allergen challenge. Ten days after intranasal administration of 10^6 BMDCs pulsed with 100 $\mu\text{g/mL}$ OVA (OVA) or pulsed with OVA (100 $\mu\text{g/mL}$) and stimulated with 100 ng/mL LPS (OVA/LPS) into naïve female BALB/c mice, allergen challenge was performed on 4 consecutive days with 50 μg OVA. On day 16, the animals were sacrificed and blood samples were collected and levels of OVA-specific IgE, IgG1, and IgG2a were determined by ELISA. Mean $\pm$ SD of 5 animals per group is shown of a representative result from a series of three independent trials.

airway reactivity after provocation with increasing doses of methacholine was increased, compared to negative controls (medium DCs). However, administration of high-IL-10-producing DCs (OVA/LPS-pulsed DCs) led to an even stronger increase in *in vivo* AR in response to unspecific airway

In vivo transfer of BMDC: AHR

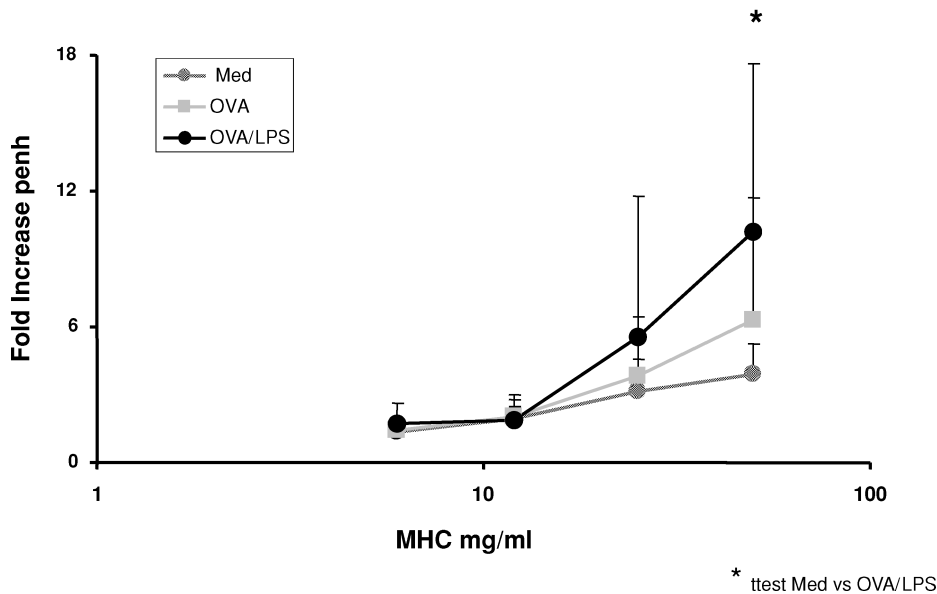


FIGURE 6 Airway reactivity after *in vivo* transfer of stimulated BMDCs and intranasal allergen challenge. BMDCs pulsed with 10^6 OVA ($100 \mu\text{g}/\text{mL}$) (OVA) or OVA-pulsed BMDCs stimulated with $100 \text{ ng}/\text{mL}$ LPS (OVA/LPS) were administered *i.n.* in naïve BALB/c mice. Unstimulated BMDCs (Medium) were used as negative control. Starting on day 10 *i.n.* antigen challenge with $50 \mu\text{g}$ OVA was performed on 4 consecutive days. Twenty-four hours after the last allergen challenge, AR measurement was performed as previously described. Fold increase of P_{enh} after 3 minutes of provocation with methacholine ($50 \text{ mg}/\text{mL}$) is shown as mean $\pm$ SD of 5 animals per group in a representative trial of a series of 3.

provocation with MCh, shown by significantly increased P_{enh} value compared to negative controls ($P = .0494$ at Mch $50 \text{ mg}/\text{mL}$) (Figure 6).

To summarize, *in vitro* we found an IL-10–dependent suppression of proinflammatory cytokine production by naïve antigen-specific CD4⁺ T cells cocultured with antigen-pulsed and LPS-stimulated BMDCs. *In vivo* the additional stimulation of BMDCs with LPS led to an even stronger eosinophilic airway inflammation and airway hyperreactivity, both typically hallmarks of an allergic inflammation. This effect was shown after administration of BMDCs treated with $10 \text{ ng}/\text{mL}$ LPS (data not shown) and after administration of BMDCs treated with $100 \text{ ng}/\text{mL}$ LPS, which produced an even higher amount of IL-10.

DISCUSSION

The aim of the present study was to delineate whether *in vitro* modulation of DC function is able to effect T-cell differentiation and thus induce tolerance in T cell–dependent immune responses *in vivo*.

Activation of DCs in the presence of OVA, regardless of further stimulation with LPS, resulted in enhanced DC maturation with selective up-regulation of surface markers CD80, CD86, CD40, and major histocompatibility complex II (MHCII). LPS treatment of DCs significantly enhanced production of IL-10 and IL-12p70 and of the proinflammatory cytokines IL-6, TNF, and IL-1 β in a dose-dependent manner (data not shown). This effect on DC maturation and function also affected subsequent T-cell functions: we observed that high-IL-10-producing DCs cocultivated with antigen-specific T cells also induced a significantly enhanced IL-10 production by T cells. In our experimental setup, these T cells were the only source for cytokine production, because they were washed and transferred to another plate 6 days after coculture, and then restimulated with PMA and ionomycin. Furthermore, these restimulated T cells produced less amounts of IL-5 and IFN- γ compared to T cells stimulated by DCs pulsed with OVA and no LPS. Interestingly, blockage and neutralization of IL-10 in LPS stimulated DCs prior to coculture with allergen-specific T cells partly restored the production of IL-5 and IFN- γ by T cells. These results strongly suggested that IL-10 was responsible for the dampening, regulatory effect on the cytokine response of the T cells. Indeed, IL-10 is known for its immune suppressive properties and is produced in high amounts, especially by regulatory T cells (Treg). At present, various types of "regulatory" T cells have been described *in vitro* or *in vivo*. They are subdivided into a number of subsets based on expression of cell surface markers, production of cytokines, and mechanisms of action [17]. In regard to our own experiments it is important to note that Tr1 cells, which belong to so-called adaptive Treg cells, develop *in vitro* in the presence of IL-10. They produce high levels of IL-10, moderate amounts of transforming growth factor (TGF)- β , IFN- γ , and IL-5, low IL-2, and no IL-4 [18]. It is still a matter of debate whether these Tr1 cells express the transcription factor Foxp3 [19]. In our own study, we did not detect a close correlation between the expression of Foxp3 (data not shown) and the magnitude of IL-10 production, although high-IL-10-producing T cells in our hands produced negligible amounts of IFN- γ and IL-5, as described for Tr1 cells.

This first set of data showed that *in vitro* stimulation of DCs with LPS had a modulating, suppressive effect, which was IL-10 dependent and resulted in the induction of a Tr1-like T-cell subset after DC/T-cell coculture.

Next we addressed the question whether these altered *in vitro* functions may be transferable into an *in vivo* model of allergic airway inflammation. We applied pulsed BMDCs into the airways of mice prior to exogenous allergen challenges in order to address the role of high-IL-10-producing DCs in allergen-specific recall and further analyzing the effects of primed DCs on naïve endogenous T cells. In our protocol we performed an intranasal application, in contrast to an intratracheal one, performed by Lambrecht and colleagues [20, 21]. The intranasal administration of OVA/LPS-pulsed DCs, which secreted high amounts of IL-10 *in vitro*, resulted in similar or even

enhanced allergic airway inflammation compared to mice receiving OVA-pulsed DC, which produced significantly less IL-10 *in vitro*. Moreover, the production of Th2-associated cytokines (IL-5 and IL-13) in the BAL fluid was more strongly enhanced after stimulation with high-IL-10-producing (OVA/LPS-pulsed) DCs, compared to low-IL-10-producing DCs, which had been matured with OVA alone. On first sight these results were contrary to the results we obtained *in vitro*, where a suppressive/regulatory effect of high-IL-10-producing DCs on T effector cells was observed. Several possibilities might explain this divergent observation, underlining important differences between *in vivo* and *in vitro* conditions: first, the *in vitro* effect might be contact dependent; where a close cell-cell contact may not be crucial *in vivo*; rather, local IL-10 concentrations differ between *in vivo* and *in vitro*. Furthermore, the exact mechanism of antigen presentation is of eminent importance for the *in vivo* situation. A recent study has shown that through repetitive respiratory exposure to allergen, high levels of IL-10-producing Treg cells were induced [22]. Therefore, we might have induced a mixed immune reaction, consisting of two different parts, one response exerted by the exogenously applied and *in vitro* generated DCs, and the other response produced by endogenous DCs reacting to the repetitive allergen airway challenge.

A third possibility to explain the discrepancy between *in vitro* and *in vivo* results is given by the fact that IL-10, although it may inhibit pulmonary inflammatory responses, under other circumstances may enhance airway hyperreactivity in allergen-sensitized mice [23]. Indeed, it was shown that IL-10 seems even to be necessary for the development of airway hyperresponsiveness, but not of pulmonary inflammation, after allergic sensitization and airway challenges [24]. This difference may reflect the importance of the timing of IL-10 administration relative to allergen sensitization and airway challenges and/or the actual time point when airway function was assessed [25]. Although we observed a regulatory effect of IL-10 on T-cell differentiation and function *in vitro*, high-IL-10-producing DCs seemed not to be able to transfer this effect *in vivo*. Of course, other mechanism besides IL-10-suppressed T-cell inhibition may be important to regulate allergen-induced airway inflammation. Tournoy and colleagues have shown that OVA-induced airway eosinophilia and IgE synthesis were suppressed in IL-10-knockout mice treated with IL-12, showing that IL-10 neither mediated nor was required for the anti-allergic effect of IL-12 [26].

In Figure 1 we have shown, that the generated DCs not only produce IL-10, but also other inflammatory cytokines, like TNF- α , IL-12p70, and IL-1 β . Although the amount of these cytokines, measured directly in the supernatant, was relatively smaller than that of IL-10, an impact on the *in vivo* lung setting is likely.

At the time point of BALF analysis, the transferred DCs (day 14 post transfer) were not detectable anymore (data not shown). Our preliminary

experiments indicated that antigen-pulsed DCs migrated to the regional lymph node very efficiently, indicating that their viability was not severely compromised. We cannot, of course, exclude that the *in vivo* environment exerted an influence that inhibited IL-10 secretion of DCs.

Interestingly, the local administration of allergen-primed DCs prior to allergen-airway challenges induced also systemic immune responses. We documented specific immunoglobulin production in the serum of animals following this protocol. After administration of high-IL-10-producing DCs, a relative increase of allergen-specific IgG2a and IgG1 was detected, whereas the application of low-IL-10-producing DCs promoted no production of OVA-specific IgG2a. This may point towards another function of IL-10 *in vivo*: It has been described that B cell-derived and exogenous IL-10 affected B-cell differentiation and isotype switching [27]. In the presence of IL-10, isotype-committed human B cells produced large amounts of IgM, IgG1-3, and IgA, and the combination of IL-10 and IL-4 induced high levels of IgG4 and IgE [28–31].

In summary, our data demonstrate that increased IL-10 production by DCs may be a critical element for T-cell activation and differentiation by down-regulation of allergen-induced immune responses *in vitro*. Divergent endogenous *in vivo* immune responses may have diminished these effects in the mouse model of allergen-induced airway disease. Thorough evaluation of the regulatory effects of IL-10 is therefore required before this approach may be applied for further treatment strategies in patients with allergic diseases.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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