

Sensitization to inhaled antigen by intratracheal instillation of dendritic cells

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Summary

Background Airway dendritic cells (DCs) capture and present inhaled antigen. It is not known whether antigen presentation by DCs in the airways is sufficient to induce sensitization to inhaled antigen *in vivo*.

Methods Rats were immunized by intratracheal instillation of ovalbumin (OVA) -pulsed bone marrow-derived DCs or macrophages and exposed 10 days later to a 30-min aerosol of OVA on 3 consecutive days. Total and differential cell counts and flow cytometry on bronchoalveolar lavage (BAL) fluid, airway histology and serum OVA-immunoglobulin (Ig) E levels were analysed 24 h after the last exposure.

Results As few as 2×10^4 OVA-DC induced sensitization to inhaled OVA. The secondary response to OVA-aerosol consisted of an antigen-specific increase in the number of bronchoalveolar mononuclear cells, activated CD4-positive $\alpha\beta$ -TCR T lymphocytes, neutrophils and few eosinophils. Peribronchial and perivascular mononuclear cell infiltrates were seen on histological analysis. There was no production of systemic OVA-IgE. Bone marrow-derived macrophages did not induce sensitization.

Conclusion Delivering antigen to the respiratory tract via professional antigen-presenting DCs sensitizes for a secondary response to inhaled antigen leading to airway inflammation. This model will prove very useful for studying the early events of sensitization to inhaled antigen using the respiratory route.

Keywords: airway inflammation, asthma, Brown Norway rat, costimulation, dendritic cell, sensitization

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Introduction

Allergic asthma is characterized by chronic eosinophilic airway inflammation, now believed to be orchestrated by T-helper 2 (TH2) lymphocytes specific for inhaled allergen [1]. T-lymphocytes respond to antigen after it has been presented in the context of major histocompatibility (MHC) molecules, a process referred to as MHC restriction. Naive T cells proliferate and differentiate in response to T-cell receptor (TCR) recognition of antigen-MHC only when accompanied by costimulatory signals (e.g. CD80 and CD86) on the surface of professional antigen-presenting cells (APCs) (reviewed in [2]). It is now clear that the

dendritic cell (DC) is the most powerful APC for inducing a primary immune response *in vivo*. It has potent antigen-capturing and presenting capacity and constitutively expresses costimulatory ligands for naive T cells (reviewed in [3]). In the bronchial epithelium, DCs form a highly developed network, projecting cell extensions between epithelial cells for maximal antigen capture [4,5]. After antigen uptake, DCs migrate to the paracortical T-cell zone of the draining lymph nodes of the lung, where they interact with naive T cells [6,7].

Few studies have addressed the question as to whether DCs can induce sensitization to inhaled antigen. Holt's group has demonstrated that epithelial and interstitial DCs of the rat internalize inhaled protein antigen efficiently *in vivo* and can present it to antigen-specific T cells *in vitro*, after an overnight maturation step [8,9]. Havenith *et al.* have

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shown that splenic DCs, but not alveolar macrophages, introduced into rat trachea, can reach the draining lymph nodes and prime naive T lymphocytes [6,10]. In these studies sensitization by DCs *in vivo* was inferred by the demonstration of enhanced proliferative T-cell responses upon *in vitro* rechallenge with antigen. In this study, we have investigated whether naive rats can be sensitized to inhaled antigen by intratracheal (i.t.) instillation of antigen-pulsed bone-marrow derived DCs. Sensitization was studied by the occurrence of a secondary inflammatory response *in vivo* upon repeated encounter with antigen aerosol.

Methods

Animals

All experiments were performed in inbred, pathogen-free, 12-week old (200–250 g) male Brown Norway (BN) rats (Harlan, Zeist, The Netherlands). Rats were housed in sterile micro-isolator units on low dust bedding (Benton-King, Hull, UK). Fischer-344 rats were used as donors for allogeneic T cells in mixed leucocyte reaction (MLR) experiments.

Monoclonal antibodies used in flow cytometry

Mouse-antirat monoclonal antibodies were used either unconjugated or conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE). The monoclonal antibodies (mAbs) OX62 (immunoglobulin [Ig] G1, anti $\alpha_E\beta_7$ integrin on DCs and epidermal $\gamma\delta$ -T cells [11,12]), OX6-PE (IgG1, anti-MHC class II molecules), OX19-FITC (IgG1, anti-CD5, a pan-T-cell marker), OX39-FITC (IgG1, anti-IL2-receptor), W3/25-FITC (IgG1, anti-CD4), OX8-FITC (IgG1, anti-CD8 α), R73-FITC (IgG1, anti- $\alpha\beta$ -TCR), OX42-PE (IgG2a, anti-CD11b [Mac-1] expressed on myeloid cells) were obtained from Serotec (Kidlington, UK) and G4.18-PE (IgG3, anti-CD3), V65-PE (IgG1, anti- $\gamma\delta$ -TCR) and isotype controls were obtained from PharMingen (San Diego, CA, USA). The mAb ED2 (IgG1, pan tissue macrophage marker) was kindly provided by Dr C. Dijkstra (Vrije Universiteit Amsterdam, The Netherlands). Unconjugated antibodies were detected with FITC-conjugated Fab fragment rat-antimouse-IgG (RAM-FITC) (Jackson Immunoresearch Laboratories, Westgrove, PA, USA).

For flow cytometry, aliquots of 2×10^5 cells were incubated with primary unconjugated or FITC-conjugated mAbs for 30 min on ice, followed by secondary labelling with RAM-FITC (if appropriate). After blocking of any residual binding of RAM-FITC with normal mouse serum, a second PE-labelled mouse antibody was added. Dead cells were excluded from analysis by labelling with propidium iodide (Sigma, St Louis, MO, USA). Three-colour fluorescence intensity analysis was performed on 1×10^4 cells on a

FACS-Vantage flow cytometer using Cellquest software. (Becton Dickinson, Mountain View, CA, USA).

Culture media and cell culture conditions

Complete medium consisted of RPMI 1640 medium supplemented with 2 mmol/L L-glutamine, 15 mmol/L HEPES buffer, 10 μ g/mL streptomycin, 100 U/mL penicillin (all from Biomedica, NTL, Brussels, Belgium) and 5% fetal calf serum (FCS; Gibco, Merelbeke, Belgium). For bone marrow culture, complete medium was supplemented with 10% FCS, 0.5 μ g/mL *n*- γ -monomethyl-L-arginine (Calbiochem, La Jolla, CA, USA), 50 μ mol/L 2-mercaptoethanol (Gibco) and 0.4 ng/mL recombinant murine granulocyte macrophage-colony stimulating factor (GM-CSF) (R & D Systems, Abingdon, UK). Cells were incubated in 75 cm² tissue culture flasks (Becton Dickinson, Erembodegem, Belgium) in a humidified incubator at 37 °C, 5% CO₂ in air.

Isolation and antigen loading of bone marrow-derived DCs and macrophages

DCs were grown from BN-rat bone marrow using a protocol described by Chenwoan *et al.* [13]. Briefly, bone marrow cells were depleted of Fc-receptor-positive cells by panning on serum-coated Petri-dishes for 1 h. Nonadherent cells were removed and cultured for 6 days in complete medium containing GM-CSF in culture flasks coated with porcine skin gelatin (Sigma). After 6 days of culture, non-adherent cells were harvested and depleted of Fc-receptor-positive adherent cells. DCs were further enriched by density centrifugation over a metrizamide gradient (Sigma; 14.5% w/v in RPMI 1640, 600 g for 20 min at 22 °C). Low density cells were incubated with the mAb OX62, expressed on the majority of bone marrow-derived DCs [13], followed by sorting of the OX62-positive population on a FACS-Vantage flow-cytometry activated cell sorter (Becton Dickinson).

For the isolation of macrophages, nonadherent bone marrow culture cells that were cultured for 6 days in medium supplemented with GM-CSF were panned on serum coated Petri dishes (as above). The adherent Fc-receptor-positive fraction was detached by gentle trypsinization (Gibco).

Purified DCs and macrophages were incubated at 1×10^6 cells/mL for 2 h in complete medium containing 1 mg/mL ovalbumin (OVA, grade V, Sigma) in a humidified CO₂ incubator. As a control, cells were incubated with 1 mg/mL of the control antigen Keyhole Limpet haemocyanin (KLH, Sigma). After this pulsing period, free antigen was removed by washing the cells five times with an excess of complete medium. Viability, as assessed by the trypan blue exclusion method, was more than 95% on each occasion.

Immunization by intratracheal instillation of OVA-pulsed DCs or macrophages

On the first day of the experiment, either 5×10^3 , 2×10^4 or 2×10^5 OVA-pulsed bone marrow-derived DCs (OVA-DC) were instilled into the airways of various groups of non-immunized rats ($n=8$ rats per group). As a control for antigen-specificity, one group ($n=8$) received 2×10^4 KLH-pulsed DCs (KLH-DC). One group ($n=7$) received 2×10^5 OVA-pulsed bone marrow-derived macrophages and one final group ($n=6$) received no primary immunization.

For the i.t. instillation of cells, rats were anaesthetized by i.p. injection of sodium pentobarbital (50 mg/kg body weight, Abbott Laboratories, Louvain la Neuve, Belgium) and an otoscope was gently introduced into the oral cavity. A cell suspension of 200 μ L was introduced through the larynx with a 19 G metal catheter connected to a micropipette.

Secondary exposure to OVA-aerosol

From days 10 to 12 after the tracheal instillation, animals were exposed daily for 30 min to an aerosol of 1% OVA in saline. Awake rats were placed in an exposure chamber connected to the outlet of an ultrasonic nebulizer that delivers an aerosol of particles with a mean diameter of 3.5 μ m (Microvernebler R80, Microvernebler, Zurich, Switzerland).

Twenty-four hours after the last aerosol exposure, rats were anaesthetized and killed by exsanguination. The trachea was surgically exposed and cannulated to perform bronchoalveolar lavage (BAL). The airways were lavaged with Ca^{2+} - and Mg^{2+} -free Hanks balanced salt solution (HBSS, Gibco, Merelbeke, Belgium), containing 0.05 mmol/L sodium EDTA. BAL fluid was centrifuged and the cells were resuspended in 1 mL ice-cold HBSS. Cells were counted in a haemocytometer (Coulter Counter ZF, Coulter, UK). Cytospin preparations of 5×10^4 cells were stained with May-Grünwald-Giemsa for differential cell counts on 300 cells.

Airway histology

After BAL was performed, fixative (4% paraformaldehyde in PBS) was gently infused through the lavage catheter by a continuous-release pump under pressure and volume-controlled conditions. The lungs were resected and fixed for an additional 4 h. After routine paraffin embedding, 4- μ m sections were stained with haematoxylin-eosin and examined by light microscopy for histological changes.

Measurement of serum OVA-specific IgE

Blood samples were taken from the sinus cavernosus for measurement of OVA-specific IgE by isotype specific

ELISA. 96-well flat-bottomed microtest plates (Nunc, Roskilde, Denmark) were incubated overnight with OVA grade V at a concentration of 50 μ g/mL in PBS, followed by incubation with 1% BSA. Serial dilutions of rat serum were applied for 1 h at 37 °C, followed by mouse-antirat-IgE (MARE, H. Bazin, Experimental immunology unit, UCL Brussels, Belgium) for 2 h at 37 °C. Bound MARE was detected by labelling with secondary horseradish peroxidase-conjugated goat antimouse immunoglobulins (1 μ g/mL) (Dako). A diaminobenzidine substrate was then applied and the plates were developed for 30 min and read at 405 nm (Titertek Multiscan MCC, Roskilde, Denmark). A serum pool of OVA-sensitized rats was used as an internal laboratory standard. Units were arbitrarily defined from optical density values from a 1/100 dilution of this pool.

Mixed leucocyte reaction (MLR)

OX62-enriched bone marrow derived DCs, bone marrow macrophages and mechanically dispersed spleen cells from BN rats were treated with 50 μ g/mL of mitomycin C (Sigma). Increasing numbers of these cells were added as stimulators to 2×10^5 nylon wool-enriched allogeneic Fischer-344 spleen T cells in round-bottom 96-well microtest plates. Five days after starting the MLR, each well was labelled with 0.5 μ Ci/mL final concentration of ^3H -thymidine (Amersham, Amersham, UK) for a 12-h period. Cells were automatically harvested (Inotech, Dottikon, Switzerland) and thymidine incorporation was determined using an automated liquid scintillation counter (1450 Microbeta Plus, Turku, Finland). Results are expressed as mean counts per minute (cpm) from triplicate wells.

Statistical analysis

All results are expressed as mean $\pm$ standard error of the mean (SEM). Comparison of means of different groups was conducted with a Mann-Whitney *U*-test [14] using the Systat statistical package (Spreadware Statistics, Palm Desert, CA, USA). Differences were considered significant at $P < 0.05$.

Results

Phenotype of transferred DCs and macrophages

Bone marrow cells were grown in GM-CSF, and nonadherent cells were collected and enriched for low-density cells over a metrizamide gradient. In pilot experiments, the phenotype of low density bone marrow cells was studied by flow cytometry. As shown in Fig. 1, more than 75% of cells expressed the DCs marker OX62. A majority of OX62-positive cells expressed the MHC class II molecule OX6 and

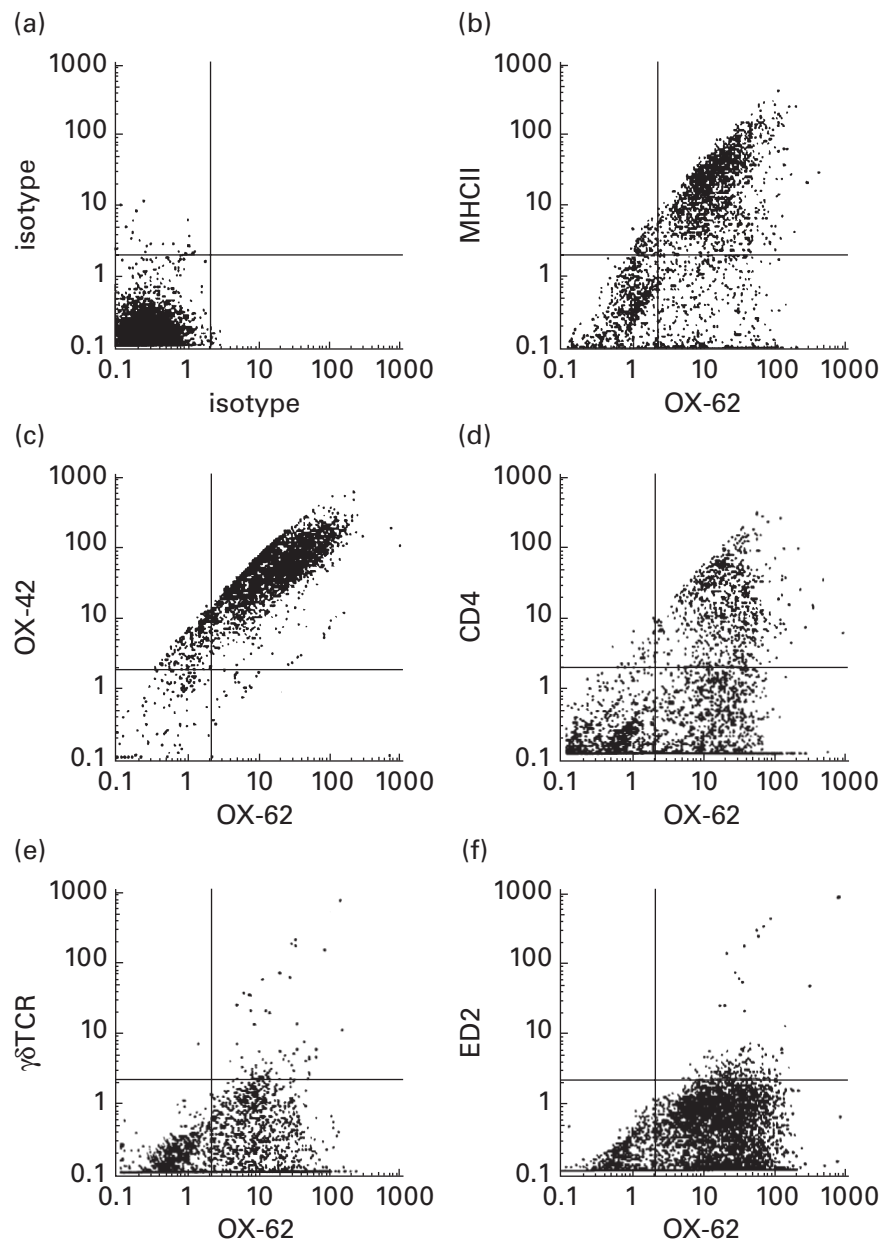


Fig. 1. Flow cytometry on cells obtained from bone marrow cultures in the presence of GM-CSF and enriched by density centrifugation over metrizamide. Cells were simultaneously stained with primary unconjugated mAb (OX62 and its isotype control) followed by secondary FITC-labelled antibodies and primary PE-conjugated antibodies as described in Materials and methods. (a) Isotype control antibodies did not stain the cells. (b) 75% of cells expressed the OX62 marker and of these, >90% strongly expressed MHC class II molecules. (c) Nearly all OX62⁺ cells co-expressed the myeloid marker OX42. (d) CD4 was expressed on almost 50% of OX62⁺ cells. Bottom: OX62⁺ cells were negative for (e) $\gamma\delta$ -TCR and (f) macrophage marker ED2

the myeloid marker OX42, whereas CD4 was expressed on approximately 50% of OX62-positive DCs. The mAb OX62 also recognizes a ligand expressed on a subset of $\gamma\delta$ -TCR T lymphocytes [11,12] but the expression of this $\gamma\delta$ -TCR on the purified OX62-positive cells was found to be negative. Purified OX62-positive DCs were negative for the tissue macrophage marker ED2. As reported by Chenwoan *et al.* and from these findings, the OX62-positive cells most likely represent the DC population among the low-density bone marrow cells, and as such DCs were further purified to >98% purity by FACS-sorting for OX62-positive cells [13]. Cytospin preparations of OX62-purified cells revealed the

typical morphology of DCs. Cells had an eccentric, large, lobulated to clover leaf-shaped nucleus, moderate to abundant grey-blue cytoplasm, and numerous long delicate processes or dendrites (Fig. 2). These highly purified DCs were used in all adoptive transfer experiments.

The Fc-receptor-positive macrophage population was highly adherent to plastic and was positive for the OX42 marker and the tissue macrophage-marker ED2 and weakly positive for MHC class II (dot plots not shown). On cytopsin preparations more than 99% of cells had an eccentric round to bean-shaped nucleus and a large vacuolated cytoplasm, typical of macrophages.

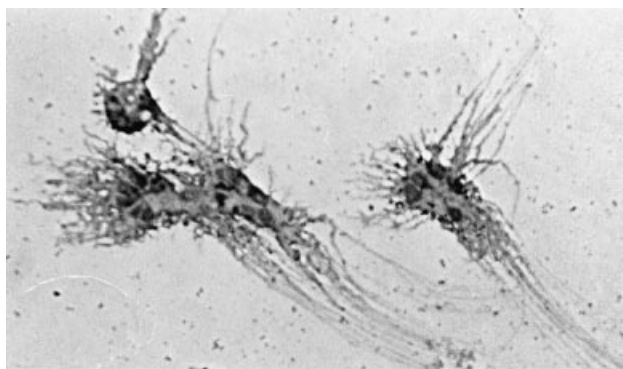


Fig. 2. Photomicrograph of purified dendritic cells. Cells were grown for 6 days in rm-GM-CSF, density enriched and sorted for the DC-specific marker OX62. (Original magnification 630 $\times$, phase contrast.)

Their capacity to strongly stimulate an allogeneic MLR is frequently used to identify DCs functionally (Fig. 3). The cells that were grown from bone marrow and sorted for OX62 expression induced a proliferation in allogeneic responder cells that extended two logs above the proliferation induced by control fresh spleen cells. Macrophages that were isolated from the same cultures and that had been grown in the medium containing GM-CSF, induced proliferation that was one log more potent than control spleen cell stimulators at low stimulatory conditions. When the number

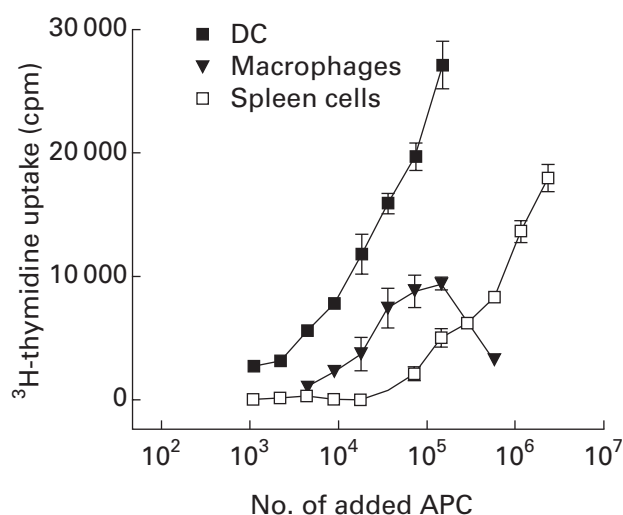


Fig. 3. Comparison of the allostimulatory capacity of fresh spleen cells with sorted BM-DC and purified macrophages in a one-way mixed leucocyte reaction. Increasing numbers of BN rat-stimulating cells were added to 2×10^5 nylon wool depleted allogeneic T cells. Data are expressed as mean [^3H]-thymidine uptake $\pm$ SEM/L of triplicate wells after 6 days of culture and are representative of three independent experiments

of macrophage stimulators was increased, however, inhibition of proliferation started to occur.

Effect of OVA aerosol exposure on BAL fluid cellular composition in rats immunized by intratracheal instillation of DCs or macrophages

As a non-specific marker for inflammation in the lungs, the total number of BAL cells was measured 24 h after the last of three exposures to OVA-aerosol in animals previously sensitized by i.t. instillation of various APCs. Preliminary experiments had shown that three exposures each 1 day apart induced a maximal cellular response in BAL fluid. The total recovery of BAL cells was significantly higher in animals immunized by i.t. administration of 2×10^4 OVA-DC compared with control antigen KLH-pulsed DCs ($P < 0.001$) (Table 1). Animals immunized with 5×10^3 OVA-DC did not show an increase in cells upon secondary challenge with OVA-aerosol compared with KLH-DC-immunized or naive rats ($P > 0.05$). The total number was significantly higher after immunization with 2×10^5 OVA-DC than after immunization with 2×10^5 OVA-pulsed macrophages ($P < 0.005$). The latter transfer did not lead to any significant increase in total cell number over the KLH-DC-induced response and was similar to the number of cells recovered from lavage in a naive animal ($P > 0.05$).

The cellular composition of BAL fluid revealed a predominance of mononuclear cells (monocytes and lymphoid cells) in naive animals, KLH-DC-immunized animals and OVA-macrophage immunized animals (Table 1). There was an increase in the number of macrophages and lymphoid-like cells in the BAL fluid of animals immunized with 2×10^4 OVA-DC compared with KLH-DC ($P < 0.005$) and the increase in these cells was even further enhanced after immunization with 2×10^5 OVA-DC. In the latter group, there was a clear increase in the number of neutrophils compared with control groups and OVA-macrophage-immunized animals. The number of eosinophils recovered by BAL after OVA-aerosol exposure was low and varied widely between animals within one group but was not influenced by immunization with any APC.

Effect of OVA aerosol exposure on the number and phenotype of lymphocytes in BAL fluid as studied by flow cytometry

To further characterize and differentiate the mononuclear and lymphoid cells readily seen on microscopical analysis of BAL fluid cells, flow cytometry was performed on BAL cells obtained 24 h after the last exposure to OVA. Analogous to reports by Lehnert *et al.* scatter profiles of unstained BAL cells revealed the presence of two dominant populations, one with the low autofluorescence and low

Table 1. Absolute numbers of total cells, macrophages, neutrophils, eosinophils and lymphoid cells in BAL fluid of animals immunized by i.t. instillation of APC, measured 24 h after the last of three OVA exposures

Immunization	Challenge	Total cells ($\times 10^6$)	Macrophages $\ddagger$ ($\times 10^6$)	Neutrophils ($\times 10^4$)	Eosinophils ($\times 10^4$)	Lymphoid cells ($\times 10^4$)
None	None	5.8 $\pm$ 0.4	5.7 $\pm$ 0.4	2.6 $\pm$ 1.9	1.8 $\pm$ 1.2	4.5 $\pm$ 2.4
2.10 ⁴ KLH-DC	OVA	4.3 $\pm$ 0.2	4.1 $\pm$ 0.2	2.1 $\pm$ 0.8	1.9 $\pm$ 2.0	13.8 $\pm$ 2.11
5.10 ³ OVA-DC	OVA	4.9 $\pm$ 0.6	4.8 $\pm$ 0.6	2.9 $\pm$ 1.7	0.3 $\pm$ 0.3	10.3 $\pm$ 5
2.10 ⁴ OVA-DC	OVA	8.3 $\pm$ 0.6 $\dagger$	7.9 $\pm$ 0.6 $\dagger$	3.0 $\pm$ 1.2	1.5 $\pm$ 1.1	33.8 $\pm$ 9.4 $\dagger$
2.10 ⁵ OVA-DC	OVA	12.2 $\pm$ 1.5*	10.7 $\pm$ 1.5*	13.3 $\pm$ 6.9*	1.7 $\pm$ 1.8	134.3 $\pm$ 23.8*
2.10 ⁵ OVA-MPh	OVA	5.5 $\pm$ 0.7	5.4 $\pm$ 0.6	1.6 $\pm$ 1.1	0	5.1 $\pm$ 3.9

* $P < 0.05$ compared with OVA-macrophages. $\dagger P < 0.05$ compared with KLH-DC. $\ddagger$ Cells were identified on cytopsin preparations stained with May–Grünwald–Giemsa.

forward- and side-scatter profile, typical of lymphocytes, the other with bright autofluorescence and large forward and side-scatter typical of alveolar macrophages and monocytes [15]. For analysis of lymphocyte subsets and activation markers, lymphocytes were gated based on these scatter characteristics.

T lymphocytes were characterized as small agranular cells, giving positive staining for both the T-cell markers CD3 and CD5 simultaneously (Fig. 4). We calculated the absolute number of BAL T cells in the different groups by multiplying the percentage of doubly positive cells by the total number of lavage cells. As seen in Fig. 5, secondary exposure to inhaled OVA induced a significant increase in the number of CD3⁺CD5⁺ T cells recovered from BAL after immunization with 2×10^4 OVA-DC compared with KLH-DC ($P < 0.001$). BAL-fluid contained a significantly higher number of T cells after immunization with 2×10^5 OVA-DC compared with equal amounts of OVA-pulsed macrophages ($P < 0.005$).

As illustrated in Fig. 4, the phenotype and activation status of the T cells in lavage was determined by flow cytometry. More than 90% of CD3⁺ T cells were CD4-positive, and 93% expressed $\alpha\beta$ -TCR, whilst no $\gamma\delta$ -TCR bearing T lymphocytes were detected. The activation status of lymphocytes can be measured by the expression of the receptor for interleukin-2 (CD25). The percentage of CD3⁺ cells expressing CD25 was significantly higher after OVA-aerosol challenge in animals immunized with 2×10^4 OVA-DC ($36.1 \pm 1.6\%$) compared with animals immunized with KLH-DC ($16.5 \pm 3.5\%$, $P < 0.005$). Activated T cells were not detected after priming with OVA-pulsed macrophages. The OX62 marker, recognizing the mucosal $\alpha_E\beta_7$ -like integrin, was detected on $47.4 \pm 1.9\%$ of T cells in the 2×10^4 OVA-DC-primed group as compared with $20.3 \pm 9.7\%$ of T cells in the 2×10^4 KLH-DC-primed group.

Effect of OVA challenge on airway histology in rats immunized by intratracheal instillation of DCs

In animals immunized with 5×10^4 OVA-DC, the 3-day exposure to OVA aerosol induced the accumulation of mononuclear infiltrates located predominantly around small blood vessels and conducting airways. Cellular infiltrates consisted of mononuclear cells, lymphocytes and a few scattered eosinophils (Fig. 6a). These changes were absent from animals immunized with KLH-DC (Fig. 6b). In some BN rats, there were scattered foci of granulomatous inflammation, irrespective of immunization status, as previously reported [16].

Effect of OVA challenge on systemic OVA-specific IgE levels in animals immunized by intratracheal instillation of APCs

In all groups of animals primed with DCs or macrophages, OVA-specific IgE immunoglobulin levels were below the detection limit (< 1.53 U/mL) of the assay (not shown).

Discussion

In this paper, we demonstrate that naive rats can be sensitized to respond to inhaled antigen by a single i.t. instillation of antigen-pulsed DCs. To obtain sufficient numbers of DCs for adoptive transfer experiments, and given the highly laborious nature of previous DC isolation procedures from airways and lung parenchyma, we modified a protocol to generate large numbers of DCs from bone marrow precursors [8,13,17,18]. Bone marrow cells were grown in GM-CSF, the predominant growth factor for DCs, and purified using the DC-specific marker OX62 [11,13]. Although this strategy seems highly artificial, DCs in the airways have a similar phenotype and are also derived from

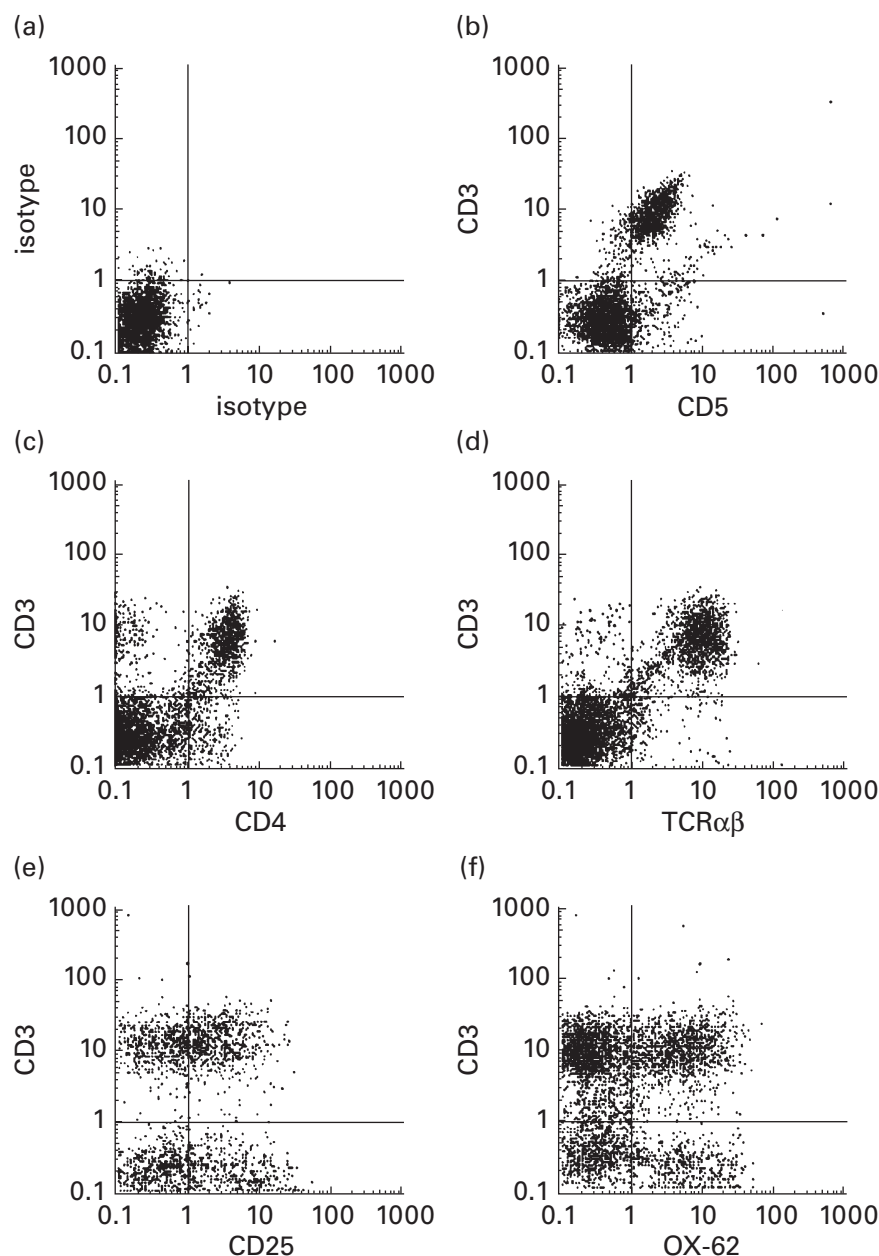


Fig. 4. Flow cytometry on cells obtained by bronchoalveolar lavage from animals immunized by i.t. injection of OVA-pulsed DCs on day 0 and challenged by daily OVA aerosol from days 10–12. Cells were gated for lymphocytes based on forward angle and sideward scatter profiles and were labelled with FITC- or PE-conjugated monoclonal antibodies as described in Materials and methods. (a) isotype-specific control antibodies were negative on cells gated for lymphocyte characteristics. (b) T cells were identified as cells that expressed both CD3 and CD5 in the lymphocyte gate. (c) 90% of CD3⁺ cells co-expressed the CD4 marker, identifying these cells as T-helper lymphocytes. (d) Nearly all CD3⁺ cells expressed the TCR $\alpha\beta$. (e) The activation marker CD25 was expressed on 36% of CD3⁺ T cells in this group (see text). (f) the $\alpha_E\beta_7$ integrin, recognized by the monoclonal antibody OX62 was expressed on almost 50% of CD3⁺ cells

a precursor in the bone marrow [4,17,19]. Functionally, these bone marrow-derived DCs were strong stimulators in an allogeneic mixed leucocyte reaction and presented soluble OVA to OVA-immune T cells (data not shown), features typical of both mature and immature DCs, respectively [3].

DCs were pulsed *in vitro* with OVA and gently introduced into the trachea through a minimally invasive technique, which did not damage the epithelial lining [20]. We chose to adoptively transfer the cells via the trachea because this is the natural route of sensitization to inhaled antigen. It was shown by Havenith *et al.* that rat spleen-derived DCs

introduced via this route reach the deep alveolar compartment of the lung [10]. The lymphatic drainage of this compartment collects into the mediastinal lymph nodes of the lung and DCs can reach these nodes 24 h after injection to interact with naive T cells [10,21].

To study sensitization to OVA by i.t. instillation of DCs, we challenged animals with OVA aerosol *in vivo* and studied the presence of a secondary response in the airways. As few as 2×10^4 OVA-pulsed DCs primed for a secondary response to challenge with OVA aerosol. The response was characterized by increased amounts of mononuclear cells, lymphocytes and neutrophils in BAL fluid. This increase in

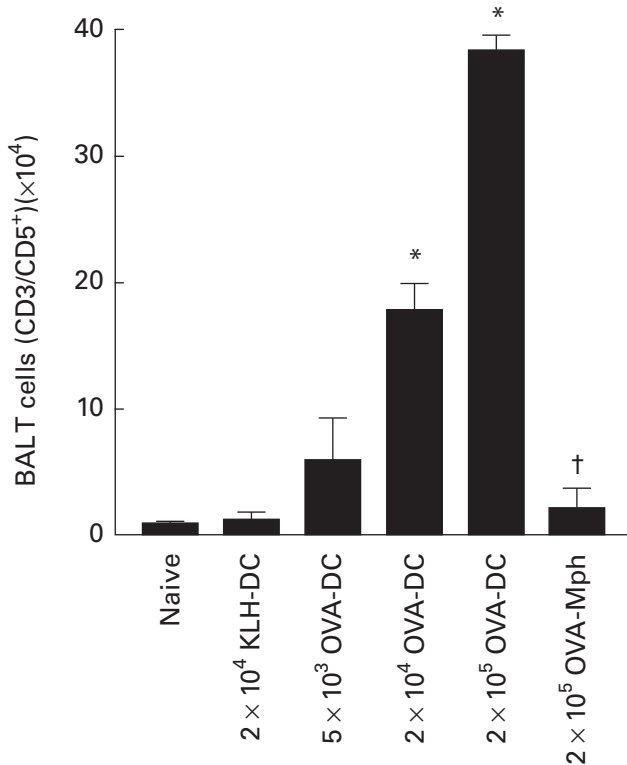


Fig. 5. Effect of secondary ovalbumin (OVA) aerosol challenge on absolute number of T cells ($CD3^+CD5^+$) recovered from BAL in animals immunized by i.t. instillation of various amounts of OVA- or KLH-DC. Animals were immunized by i.t. injection on day 0 and challenged by daily aerosol from days 10–12. Results are mean values from six to eight animals per group. (* $P < 0.005$ vs 2.10^4 PBS-DC, † $P < 0.005$ vs 2.10^5 OVA-DC)

inflammatory cells in BAL fluid was a reflection of the development of perivascular and peribronchial mononuclear cell infiltrates, readily noticed on histological analysis. The response was antigen specific as KLH-pulsed DCs did not sensitize for a secondary response to inhaled OVA. Increasing the number of transferred DCs to 2×10^5

cells/animal induced a stronger response, illustrating the dose–response relationship of the response. Flow cytometric analysis on BAL fluid revealed that the induced T cells were of the activated ($CD25^+$) $CD4^+$ T-cell type, expressing the $\alpha\beta$ -TCR. It was shown by Wanatabe *et al.* that $CD4$ -positive cells, purified from rats immunized by i.p. injection of OVA in adjuvant, orchestrate airway inflammation in response to OVA challenge [22]. As we have not performed adoptive transfer experiments of these $CD4^+$ T cells in our model, we can only speculate that they are similarly important for controlling the inflammatory response. One striking finding was the expression of the $\alpha_E\beta_7$ -integrin, recognized by the OX62 antibody, on these $\alpha\beta$ -TCR-positive T cells in BAL fluid [11,12]. The expression of this integrin had previously only been demonstrated on intraepithelial and afferent lymphatic DCs and $\gamma\delta$ -TCR T cells of the rat. This integrin is important for retention of lymphocytes at epithelial linings and has been specifically implicated as a marker for human and rat T cells in the alveolar lumen [18,23,24].

We have compared the immunostimulatory capacity of DCs with macrophages. Although alveolar macrophages are easily obtained by BAL, they are poor APCs and can even downregulate T-cell immune responses [8,25]. Therefore, macrophages were grown from the same bone marrow precursors as DCs and exposed to GM-CSF under identical conditions. Cytokines, such as GM-CSF and $IFN\gamma$ have potent stimulatory activity on macrophages and can turn a suppressive macrophage into an efficient APC [26]. However, up to 2×10^5 OVA-pulsed GM-CSF-pretreated macrophages were unable to induce any significant sensitization. In line with these findings, i.v. injection of inflammatory peritoneal macrophages did not induce sensitization *in vivo* [27]. A possible explanation for this could be a lack of costimulatory activity [28]. The costimulatory activity of the purified DCs was about 10-fold higher than the purified macrophages *in vitro*, but the response induced by 10-fold higher numbers of macrophages (2×10^5) was still insignificant compared with the response induced by 2×10^4 DCs,

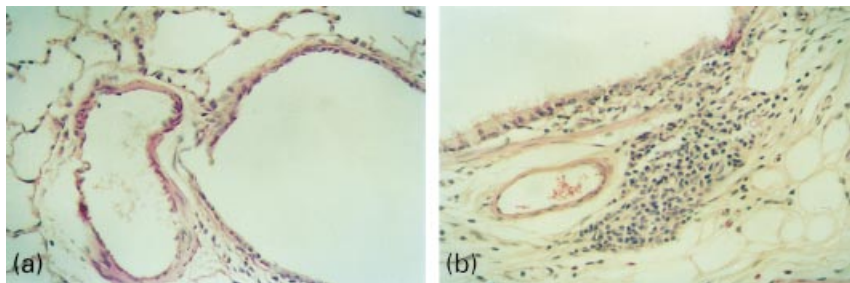


Fig. 6. Effect of secondary OVA aerosol exposure on lung histology. Animals were immunized on day 0 by intratracheal instillation of DCs and exposed from day 10–12 to daily 30 min OVA-aerosol. Lungs were resected 24 h after the last exposure, processed for paraffin sectioning and stained with H & E. Original magnification 400 $\times$. (a) Rat immunized with 5×10^4 KLH-pulsed DCs and exposed to OVA-aerosol. (b) Rat immunized with 5×10^4 OVA-pulsed DCs and exposed to OVA-aerosol

suggesting that the absence of costimulation was not the only reason for the lack of response. A failure to migrate to the draining lymph nodes of the lung, critical for the initiation of the primary immune response, could also be a contributing factor [6]. In contrast with our findings, prior studies have found some degree of sensitization by i.t. administration of alveolar macrophages [6,29].

We and others have never observed secondary inflammatory responses to inhaled OVA in animals that were previously immunized by OVA aerosol only [30,31]. Repeated exposure of naive rats to non-pathogenic protein antigens induces a state of T-cell-mediated immunological tolerance [32,33], although Gelfand's group found secondary responses in mice sensitized by respiratory route only. These responses consisted primarily of bronchial hyperreactivity and short-lived production of specific IgE in the absence of overt inflammation and were primarily seen in genetically prone animals [34,35]. In our previous experiments, systemic sensitization by i.p. injection of OVA in adjuvant was necessary for the induction of OVA-induced airway inflammation [36,37]. In the present experiments, however, we induced sensitization via the respiratory route by targeting OVA to the most relevant APCs of the primary immune response and injecting these cells into the trachea. As the injection of a bolus of purified DCs is a rather artificial strategy, we can only speculate that resident DCs of the lung are similarly important for sensitization to naturally inhaled antigen. Holt's group has shown that epithelial and interstitial DCs internalize inhaled protein antigen *in vivo* and can present it to antigen-specific T cells *in vitro* [8,9]. The immunostimulatory function and degree of maturation of airway DCs *in situ* is held under strict control by suppressive macrophages [8,9,25]. By injecting mature DCs into the deeper compartments of the lung, we have bypassed this regulatory control. However, the inhibitory effect of alveolar macrophages can also be temporarily suppressed by inflammatory stimuli, so that resident and newly attracted DCs would indeed induce sensitization [9,26,38,39]. Recently, Stampfli *et al.* induced sensitization and airway inflammation by OVA aerosol exposure in mice infected with an adenovirus expressing GM-CSF in the airways. The high levels of GM-CSF led to an increase in the number and stimulatory function of airway DCs. Presumably, T cells were activated and tolerance was avoided, illustrating that endogenous airway DCs can induce sensitization, given a stimulus for maturation [31].

The response to OVA aerosol in OVA-DC-immunized BN-rats was characterized by the absence of systemic OVA IgE production and airway eosinophilia. This was unexpected as the BN rat is a high IgE responder and is spontaneously skewed towards the development of tissue eosinophilia and TH2 responses [40]. It could be that by targeting the antigen intracellularly to DCs, there was no

free antigen remaining for stimulation of the B-cell receptor, and no B-cell response was induced [41]. As cytokine levels in BAL fluid were unmeasurable in our studies, we can only speculate as to the type of TH-response that was induced. Although the absence of IgE production and tissue eosinophilia would suggest that the T cells were of TH1 phenotype, it is possible that under different experimental conditions, a TH2 response with tissue eosinophilia would be more prominent [42,43]. The i.p. injection of heat killed *Bordetella pertussis* in alum at the time of i.t. instillation of DCs increased the proportion of eosinophils in BAL fluid suggesting that the sensitization induced by DCs can be modified by exogenous factors [44].

In conclusion, DCs can induce sensitization to inhaled antigen. These results and our findings that DCs are essential for the maintenance of eosinophilic airway inflammation suggest that DCs are critical cells in the pathogenesis of asthma [5,18,45]. This model should further allow us to study the nature of sensitization and to alter its outcome.

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