

Concomitant Inhalation of Cigarette Smoke and Aerosolized Protein Activates Airway Dendritic Cells and Induces Allergic Airway Inflammation in a TLR-Independent Way¹

Lander J. Robays,^{2,*†} Ellen A. Lanckacker,^{2,*} Katrien B. Moerloose,* Tania Maes,* Ken R. Bracke,* Guy G. Brusselle,* Guy F. Joos,* and Karim Y. Vermaelen^{3,*}

Cigarette smoking is associated with the development of allergic asthma. In mice, exposure to cigarette smoke sensitizes the airways toward coinhaled OVA, leading to OVA-specific allergic inflammation. Pulmonary dendritic cells (DCs) are professional APCs involved in immunosurveillance and implicated in the induction of allergic responses in lung. We investigated the effects of smoking on some of the key features of pulmonary DC biology, including trafficking dynamics and cellular activation status in different lung compartments. We found that cigarette smoke inhalation greatly amplified DC-mediated transport of inhaled Ags to mediastinal lymph nodes, a finding supported by the up-regulation of CCR7 on airway DCs. Pulmonary plasmacytoid DCs, which have been involved in inhalational tolerance, were reduced in number after smoke exposure. In addition, combined exposure to cigarette smoke and OVA aerosol increased surface expression of MHC class II, CD86, and PDL2 on airway DCs, while ICOSL was strongly down-regulated. Although inhaled endotoxins, which are also present in cigarette smoke, have been shown to act as DC activators and Th2-skewing sensitizers, TLR4-deficient and MyD88 knockout mice did not show impaired eosinophilic airway inflammation after concomitant exposure to cigarette smoke and OVA. From these data, we conclude that cigarette smoke activates the pulmonary DC network in a pattern that favors allergic airway sensitization toward coinhaled inert protein. The TLR independency of this phenomenon suggests that alternative immunological adjuvants are present in cigarette smoke. *The Journal of Immunology*, 2009, 183: 2758–2766.

Increasing epidemiological and experimental data now support an active role of cigarette smoking in the development and severity of asthma (1, 2). Active smoking interacts with the asthmatic phenotype, causing more severe allergic symptoms, a greater decline in lung function, and impaired therapeutic responses to corticosteroids (3, 4). In addition, smoke exposure is considered as a risk factor for allergen sensitization and is associated with the onset of asthma (5–7).

To better understand these effects, animal models studying the interaction between cigarette smoke and allergic responses have been developed (8–16). We and others recently established a mouse model in which concomitant inhalation of aerosolized OVA along with mainstream cigarette smoke, without prior immunization, induces features of allergic airway inflammation (16, 17). This allergic response was absent after exposure to OVA alone, suggesting a prominent role for cigarette smoking in the establishment of an OVA-specific Th2 immune response. Indeed, synthesis

of IL-5, a Th2-derived cytokine critical for the recruitment and survival of eosinophils, was detected in thoracic lymph node (LN)⁴ cell suspensions of OVA/smoke-exposed animals only. However, not all studies supported a positive contribution of cigarette smoke exposure to the allergic phenotype and sensitization phenomenon, possibly as a result from the variable experimental conditions used among different laboratories (e.g., the use of environmental vs mainstream smoke, whole-body vs nose-only exposure, etc.) (11, 12, 14, 15). One outstanding observation was that the effects of smoke exposure on allergic responses appear to be dose dependent (15).

Pulmonary dendritic cells (DCs) are specialized APCs and have emerged as central players in the immunological balance of the airways (18, 19). A continuous flow of migrating DCs interacting with resident airway cells constantly assesses whether inhaled material should be confronted with an active immune response or whether homeostatic tolerance should be maintained (20). The outcome of adaptive immune responses depends on the context in which the Ag is encountered by airway DCs and is predominantly defined by the presence or absence of danger signals at the exposed site, such as pathogen-associated molecular patterns betraying the presence of bacterial, fungal, or viral pathogens and damage-associated molecular patterns released by stressed or damaged host tissue cells (21–23). TLRs expressed on DCs are essential for the

*Department of Respiratory Medicine, Ghent University Hospital, Ghent, Belgium; and [†]Laboratory of Molecular and Cellular Therapy, Department of Physiology and Immunology, Medical School, Vrije Universiteit Brussel, Belgium

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² L.J.R. and E.A.L. contributed equally to this manuscript.

³ Address correspondence and reprint request to Dr. Karim Vermaelen, Department of Respiratory Medicine, Ghent University Hospital, De Pintelaan 185, 9000 Ghent, Belgium. E-mail address: karim.vermaelen@ugent.be

⁴ Abbreviations used in this paper: LN, lymph node; DC, dendritic cell; AW-DC, airway-derived DC; BAL, bronchoalveolar lavage; pDC, plasmacytoid DC; TSLP, thymic stromal lymphopoietin; MHCII, MHC class II; PAS, periodic acid-Schiff; MFI, mean fluorescence intensity; PDL, programmed death ligand.

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integration of these alarm signals and different pulmonary DC subsets have been shown to express different TLRs, potentially leading to different immunological outcomes (24). Triggering of TLRs leads to DC activation manifested by the up-regulation of a specific set of costimulatory molecules, enhanced migration toward draining LNs, and efficient priming of T cells, which will recirculate to the periphery in an attempt to neutralize the threat. In the setting of allergic airway inflammation, DCs mount an inappropriate Th2-type inflammatory immune response against otherwise nonpathogenic inhaled material (25, 26). The immunological mechanisms by which DCs induce this aberrant response are being unraveled. A working hypothesis is that environmental factors acting as damage-associated molecular patterns affect airway DCs in a way that breaks tolerance toward coinhaled inert Ag (18, 23). In this study, we asked ourselves whether cigarette smoke inhalation affects pulmonary DCs in a way that would facilitate allergic sensitization to coinhaled OVA, a model inert protein Ag. Because TLR triggering by trace amounts of endotoxin has been shown to spark allergic sensitization to OVA (27–29) and because cigarette smoke has been shown to contain bioactive endotoxins (30), we verified whether TLR4 and MyD88-dependent TLR signaling contributes to the phenomenon of smoke-induced allergic sensitization.

Materials and Methods

Mice

BALB/c mice (7–9 wk old) were obtained from Harlan. TLR4-deficient mice (C3-*Tlr4*Lps-d/J, 8–12 wk old) and control BALB/c mice (7–9 wk old) were purchased from The Jackson Laboratory. MyD88 knockout mice on a BALB/c background were obtained from the laboratory of G. Lauvau (INSERM, University of Sofia Antipolis, Valbonne, France), bred in our facility, and 6- to 8-wk-old mice were provided by Dr. O. Krysko (Ear-Nose-Throat Research Laboratory, Ghent University Hospital, Ghent, Belgium). Control BALB/c mice were purchased from Harlan. The local Ethical Committee (Ethics Committee, Faculty of Medicine and Health Sciences, Ghent, Belgium) approved all in vivo manipulations.

Cigarette smoke exposure and aerosol challenge

Groups of seven to eight mice were subjected to whole-body mainstream cigarette smoke exposure of five Kentucky Reference cigarettes (2R4F without filters; University of Kentucky, Lexington, KY) for 7 min, 4 times/day, 5 days/week in a Plexiglas chamber (17 × 28 × 14 cm) with an inlet for pressurized air (1.25 liter/min). For the MyD88 experiment, 3R4F Kentucky cigarettes were used. In the experiments where cigarette smoke and aerosol exposure were performed simultaneously, the smoking chamber was additionally connected to an ultrasonic aerosol generator. Concurrent aerosol challenge with PBS or 1% OVA (grade III; Sigma-Aldrich) in PBS was performed for 7 min, 4 times/day, 5 days/week. The amount of contaminating LPS found in the grade III OVA preparation was measured by the *Limulus* amoebocyte lysate assay and determined at 5.82 ng of bioactive LPS/mg of protein.

Bronchoalveolar lavage (BAL) and cytopspins

Twenty-four hours after the last exposure, mice were sacrificed by a lethal dose of pentobarbital. A tracheal cannula was inserted and BAL was performed by instillation of 3 × 300 μ l of HBSS supplemented with BSA for cytokine analysis. Three additional instillations with 1 ml of HBSS plus EDTA were performed to achieve maximal recovery of BAL cells. Five thousand BAL cells were processed for cytopspins and were stained with May-Grünwald-Giemsa for differential cell counting. The remaining cells were used for FACS analysis.

Preparation of single-cell suspensions

Lungs were perfused with saline plus EDTA through the pulmonary artery to remove contaminating blood cells. Lungs and mediastinal LNs were removed and digested as detailed previously (31). Briefly, minced lung pieces and LNs were incubated with 1 mg/ml collagenase and 20 μ g/ml DNase I for 45 min at 37°C. RBC were lysed using ammonium chloride buffer. Finally, cell suspensions were filtered through a 50- μ m nylon mesh to remove undigested organ fragments.

Flow cytometry

All staining procedures were conducted in calcium- and magnesium-free PBS containing 10 mM EDTA, 1% BSA (Dade Behring), and 0.1% sodium azide. One million cells were preincubated with anti-CD16/CD32 (2.4G2) to block Fc receptors. mAbs used to identify cell surface molecules were anti-CD11c (clone HL3), anti-CD11b (clone M1/70), anti-MHC class II (MHCII; I-A/I-E, clone M5/114), and anti-CD86 (clone GL1), all from BD Biosciences; anti-ICOSL (anti-CD275, clone HK5.3), anti-PDL1 (anti-CD274, clone MIH5), anti-PDL2 (anti-CD273, clone 122), anti-OX40L (anti-CD134L, clone RM134L), anti-GITRL (clone YGL386), and anti-CCR7 (CD194, clone 4B12) were from eBioscience; and the PDCA-1 mAb (clone JF05-1C2.4.1) was from Miltenyi Biotec.

Evaluation of dendritic cell migration by intratracheal instillation of fluorescent OVA

After 3 wk of smoke exposure, mice were anesthetized with Avertin (1 mg of tribromethanol/ml *t*-amylalcohol in 2.5% in PBS). The dose of the Avertin solution was carefully determined according to the body weight of each mouse separately so that no respiratory depression was observed. Seventy microliters of fluorescein-conjugated OVA (OVA-FITC; Invitrogen) diluted in sterile PBS (5 mg/ml) was injected intratracheally using pyrogen-free catheters in order not to cause any local pulmonary inflammation, as described previously (31). Mice were further exposed to cigarette smoke until they were sacrificed at indicated time points after instillation. Mediastinal LNs were removed and processed as described above to obtain single-cell suspensions. The number of OVA-bearing airway-derived DCs (AW-DC) was calculated by flow cytometry as the fraction of FITC-positive CD11c⁺MHCII^{high} LN cells multiplied by the total number of LN cells. PBS-instilled mice were used as a control to eliminate background fluorescence.

Histology

Left lung was fixed in 4% paraformaldehyde and embedded in paraffin. Transverse sections of 3 μ m were stained with Congo Red (Klinipath) for the visualization of eosinophils or with periodic acid-Schiff (PAS) to identify mucopolysaccharides in tissue and counterstained with hematoxylin (Sigma-Aldrich). PAS-positive cells were identified as goblet cells and quantified in airway walls with a perimeter basement membrane between 800 and 2000 μ m.

Measurement of OVA-specific IgE

Blood was collected by cardiac puncture for measurement of total and OVA-specific IgE with a homemade ELISA as described before (17).

RNA extraction and RT-PCR

RNA from lung tissue was extracted using a Qiagen RNeasy Mini Kit. Real-time RT-PCR was performed on a LightCycler 480 Instrument (Roche Diagnostics) with Assays-on-Demand Gene Expression Products (Applied Biosystems) starting from 20 ng of cDNA. Monitoring of the RT-PCR occurred in real time using a FAM/TAMRA probe.

For IL-13, reverse transcription was performed for 10 min at 25°C, 60 min at 42°C, and 10 min at 70°C using random hexamers and RevertAid M-MuLV Reverse Transcriptase (Fermentas). After a 10-min incubation at 95°C for denaturation of RNA-DNA heteroduplexes, a DNA amplification was performed with 50 cycles of 95°C for 15 s and 60°C for 60 s using the TaqMan Universal Primer Mix No AmpErase UNG (Applied Biosystems). Expression of the mRNA of the gene of interest relative to *hpr* mRNA was calculated.

Statistical analysis

Reported values are expressed as mean $\pm$ SEM. Statistical analysis was performed with SPSS software (version 15.0) using nonparametric tests (Kruskal-Wallis and Mann-Whitney *U* test). Values of $p < 0.05$ were considered significant (*, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$).

Results

Simultaneous exposure to cigarette smoke and OVA induces allergic airway inflammation

The effect of cigarette smoking on the development of allergic responses was illustrated by exposing mice to a combination of OVA aerosol and cigarette smoke without prior immunization to OVA (17). As we observed earlier, 3 wk of simultaneous exposure induced features of allergic airway inflammation, demonstrated by accumulation of eosinophils, neutrophils, and lymphocytes and in

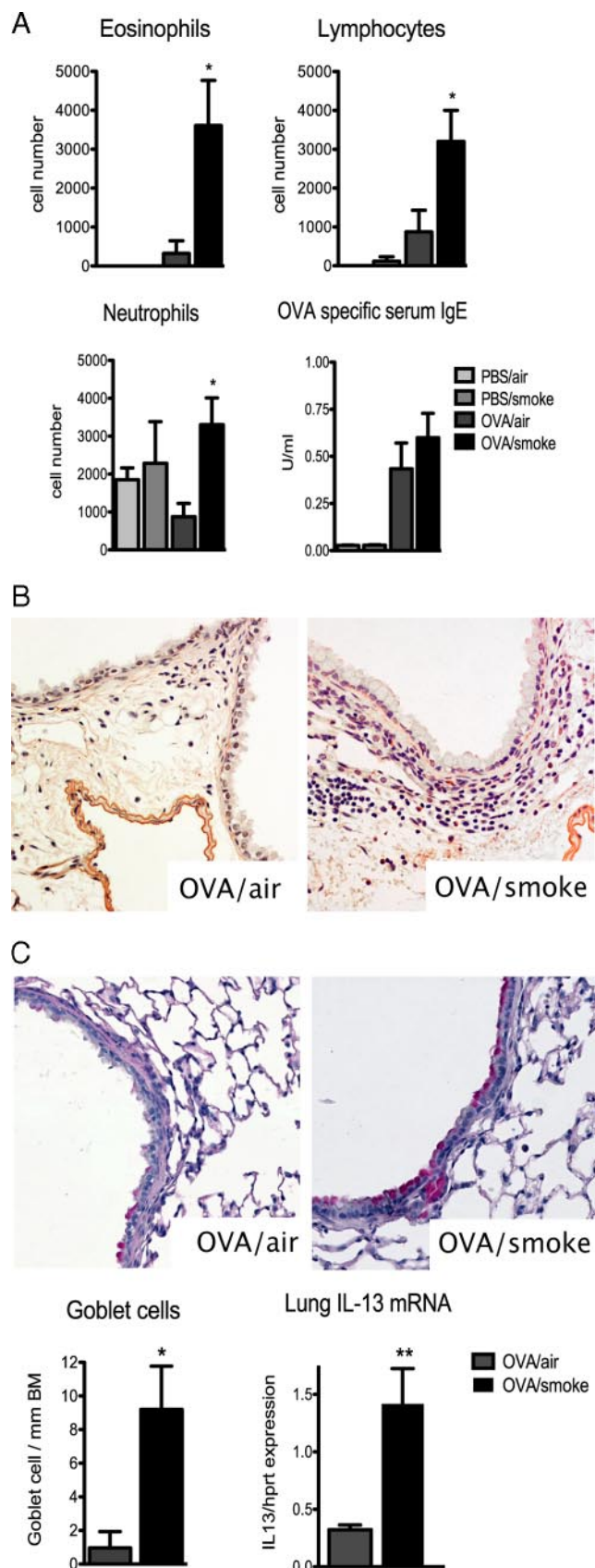


FIGURE 1. Allergic sensitization of mice exposed to cigarette smoke and OVA. **A**, Absolute numbers of eosinophils, lymphocytes, and neutrophils in BAL fluid and serum OVA-specific IgE of mice exposed to PBS or OVA combined with cigarette smoke or air for 3 wk. Cells were identified on cytospins stained with May-Grünwald-Giemsa. The concentration of OVA-specific IgE in serum was determined by ELISA. **B**, Histological

the BAL fluid (Fig. 1A), production of OVA-specific IgE in serum (Fig. 1A), eosinophilic infiltrates in the peribronchial tissue (Fig. 1B), and marked airway goblet cell hyperplasia on histological airway sections (Fig. 1C), fully consistent with earlier observations in this model (17). In line with the goblet cell hyperplasia, we observed a strong induction of pulmonary IL-13 mRNA expression in mice concomitantly exposed to OVA and cigarette smoke compared with OVA alone (Fig. 1C). We also showed earlier that this allergic response was associated with OVA-specific Th2 cytokine production in the draining LNs (17). Taken together, this suggests that cigarette smoke possessed adjuvant properties toward concomitantly inhaled OVA that favored the development of mucosal Th2 responses.

Effects of cigarette smoke exposure on pulmonary DC dynamics and Ag transport

Pulmonary DC trafficking can be divided into a recruitment phase toward the lung, progressing to an emigration phase toward mediastinal LNs. Combined exposure to cigarette smoke and OVA induced a strong elevation in conventional DC numbers in the BAL compartment, indicating that the developing allergic inflammation in these mice stimulated DC recruitment to the airways (Fig. 2A), which is consistent with earlier observations using i.p. alum as an adjuvant (32). Airway Ag-specific T cell priming relies on the capture and transport of inhaled Ag by DCs migrating to the mediastinal LNs. Hence, we investigated whether smoke inhalation as such could affect airway DC trafficking and Ag transport. We delivered fluorescently labeled OVA into the airways once after 3 wk of smoke exposure alone. At various time points after intratracheal challenge, FITC⁺ cells were detected within CD11c⁺ MHCII^{high} AW-DCs in the mediastinal LNs according to previous studies (31) (Fig. 2B, upper graph). The time course of these FITC⁺ AW-DCs in LNs of sham-exposed animals illustrated steady-state kinetics characterized by a peak of DCs infiltrating the LNs at 24 h after Ag challenge, slowly declining thereafter (Fig. 2B, lower graph). By contrast, smoke-exposed animals showed a marked amplification of DC-mediated Ag transport to LNs, i.e., more Ag-loaded DCs reaching the draining LNs (Fig. 2B, lower graph). This large wave of DCs carrying Ags to the LNs during smoke exposure peaked at 24–48 h after Ag challenge and rapidly declined thereafter. Accordingly, we found that cigarette smoke exposure selectively up-regulated surface expression of CCR7 on airway DCs, i.e., the population primarily involved in airway Ag capture (Fig. 2C). No CCR7 could, however, be detected on lung parenchymal DCs.

Next to the striking effects on conventional DCs in the lung, cigarette smoke inhalation also affected pulmonary plasmacytoid DCs (pDCs). Pulmonary pDCs were shown to actively contribute to tolerance toward inhaled Ag (33). Remarkably, the numbers of CD11c^{int}MHCII^{low/int} PDCA-1⁺ cells (generally regarded as pDCs) were reduced both within lung and mediastinal LNs of cigarette smoke-exposed mice (Fig. 2D).

evaluation of eosinophilic peribronchial infiltrates on Congo Red-stained lung tissue sections counterstained with hematoxylin. **C**, Histological evaluation of goblet cell hyperplasia on PAS-stained lung tissue sections. Quantification of the number of goblet cells per mm perimeter basement membrane (BM) of airway walls with a perimeter between 800 and 2000 μ m of mice exposed to OVA with or without cigarette smoke. mRNA transcripts for IL-13 relative to a housekeeping gene (*hprt*) were measured in whole lung homogenates by real-time RT-PCR. A representative experiment ($n = 8$ mice/group) of three independent experiments is shown. *, A significant difference ($p < 0.05$) between the OVA/air and OVA/smoke group.

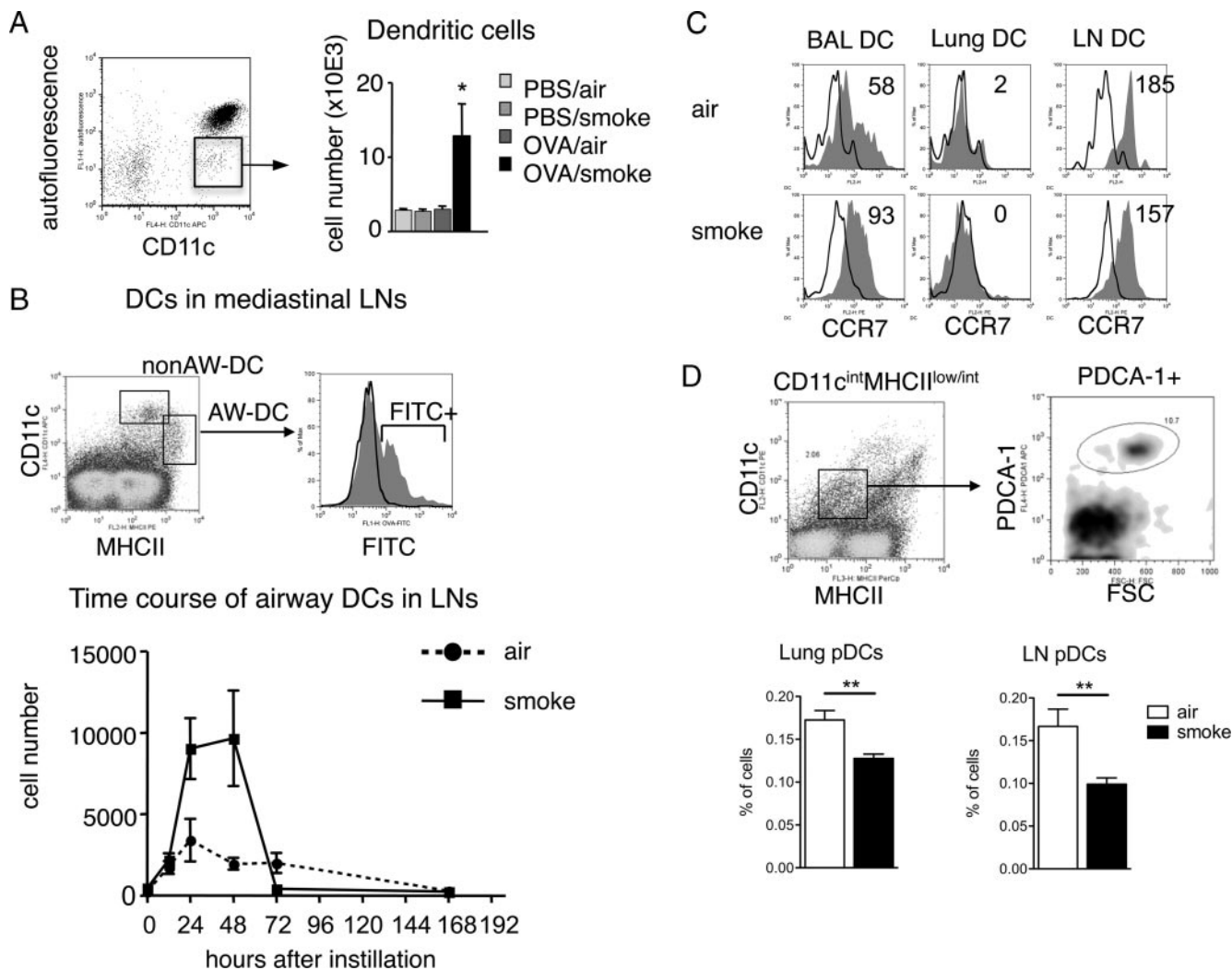


FIGURE 2. Migration of pulmonary DCs during cigarette smoke exposure. **A**, Numbers of DCs in the BAL of mice exposed to a combination of smoke and aerosolized OVA for 3 wk. DCs were identified as low-autofluorescent CD11c^{high} cells in the cellular fraction of BAL fluid. *, A significant difference ($p < 0.05$) between the OVA/air and OVA/smoke group. **B**, Kinetics of DC emigration to the draining LNs. Mice were exposed to mainstream cigarette smoke (smoke) or sham exposed (air). After a 3-wk exposure, 70 μ l of an OVA-FITC solution in sterile PBS was instilled into the airways and mediastinal LNs were isolated at various time points after instillation. Single-cell suspensions were prepared from LNs by enzymatic digestion and labeled with CD11c and MHCII. FITC⁺ cells were exclusively found within the fraction of CD11c⁺MHCII^{high} AW-DCs. Graph depicts the time course of absolute numbers of FITC⁺ AW-DC at various time points after instillation between smoke-exposed mice (smoke) and sham-exposed mice (air). **C**, CCR7 expression on pulmonary DCs from 3-wk cigarette smoke-exposed mice (smoke) and sham-exposed mice (air). Single-cell suspensions derived from BAL fluid and enzymatically digested lung tissue and mediastinal LNs were stained with mAbs for CD11c, MHCII, and CCR7. DCs were identified as low-autofluorescent CD11c^{high} cells in BAL fluid (BAL DC) and lung tissue (lung DC) and as CD11c⁺MHCII^{high} cells in LNs (LN DC). Representative histogram of CCR7 expression on DCs is shown (gray shaded) relative to isotype control staining (black line). Cells of three mice ($n = 9$ mice/group) were pooled before staining. **D**, Gating strategy for pDCs characterized as CD11c^{int}MHCII^{low/int} PDCA-1⁺ cells. Bar graphs indicate the numbers of pDCs in lung digests and mediastinal LNs of smoke- vs air-exposed mice (for 4 wk). FSC, Forward scatter.

Effects of inhaled cigarette smoke and/or aerosolized OVA on pulmonary DC activation and costimulatory molecule expression

Costimulatory molecules expressed on DCs during Ag presentation contribute to the outcome of T cell stimulation and polarization. We therefore examined expression levels of various costimulatory molecules on the cell surface of pulmonary DCs during smoke and/or OVA Ag exposure. We focused on accessory molecules with documented involvement in the establishment of tolerance vs Th2 development. Examination of the expression levels of MHCII and CD86 showed that the majority of pulmonary DCs from the airway compartment and lung tissue of OVA/smoke-exposed mice exhibited a mature or activated phenotype (Fig. 3). This was also observed on DCs from

PBS/smoke-exposed mice in the airway compartment and peripheral lung tissue, consistent with our previous results (34, 35). Expression of ICOSL (CD275), a member of the B7 family and a negative regulator of immune responses (36), was down-regulated from the cell surface of airway and lung DCs of OVA/smoke-exposed DCs compared with other groups. Alternatively, expression of two other B7 family members, programmed death ligand (PDL) 1 (CD274) and PDL2 (CD273), both ligands for the inhibitory TCR programmed death receptor 1 (PD1, CD279), was increased on DCs from OVA/smoke-exposed mice in the airway compartment and lung tissue. Remarkably, PDL2 was clearly up-regulated on AW-DCs in mediastinal LNs of combined OVA/smoke- and PBS/smoke-exposed mice. We were not able to detect significant amounts of GITRL or

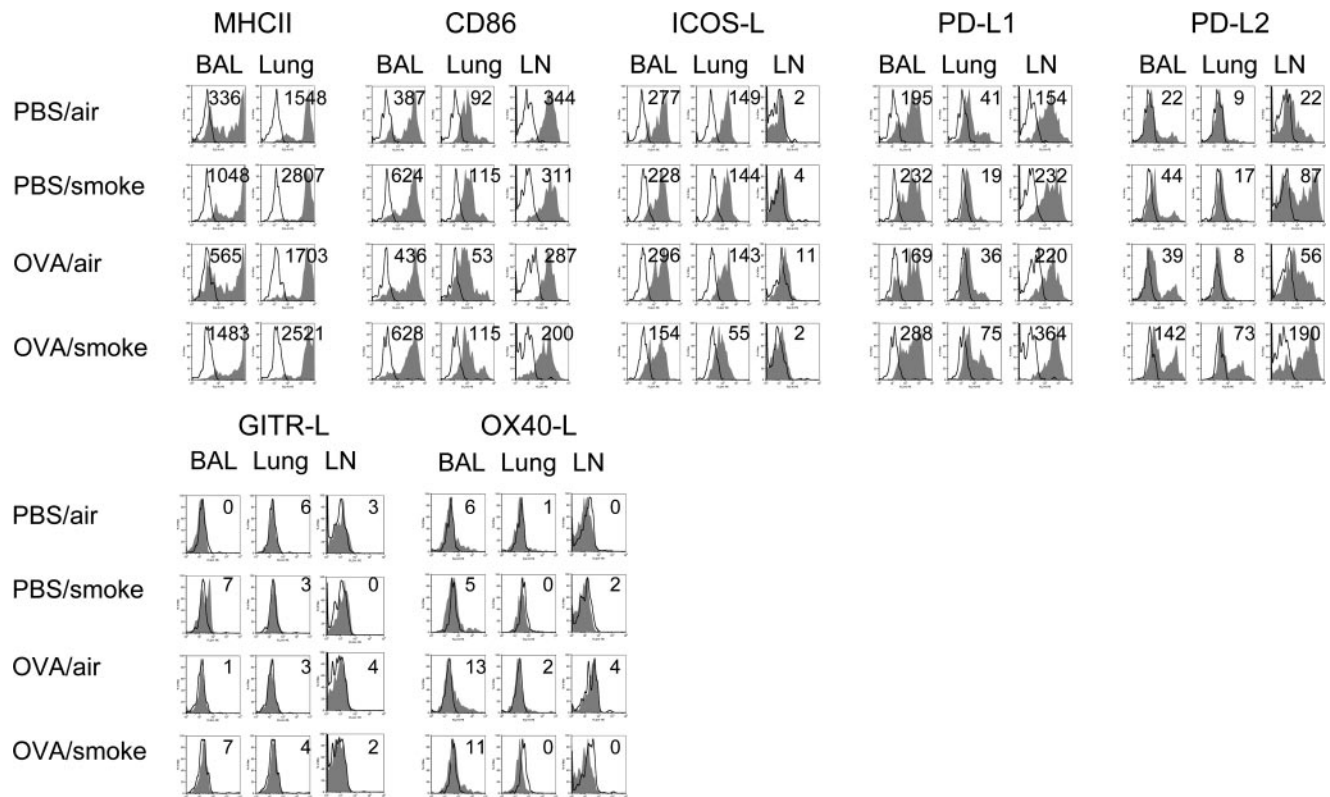


FIGURE 3. Costimulatory molecule profile of pulmonary DCs during cigarette smoke and/or aerosolized OVA exposure. Mice were exposed to cigarette smoke (smoke)- or sham-exposed (air) along with a PBS or OVA aerosol for 3 wk. Single-cell suspensions were prepared from BAL fluid and enzymatically digested lung tissue and mediastinal LNs and stained with mAbs. DCs were identified as low-autofluorescent CD11c^{high} cells in BAL fluid (BAL DC) and lung tissue (lung DC) and as CD11c⁺MHCII^{high} cells in LNs (LN DC). Expression levels of various costimulatory markers (gray histograms) are shown relative to isotype control staining (black line). Relative MFI (Ab corrected for MFI isotype control) is indicated. Cells of three mice ($n = 9$ mice/group) were pooled to obtain sufficient cells for staining. Representative histograms of two independent experiments are shown.

OX40L (CD134L) on pulmonary DCs by flow cytometry (compared with OX40L-positive control staining on splenocytes stimulated with LPS; data not shown).

TLR independency of cigarette smoke-induced allergic sensitization

TLR4 has previously been reported to play a pivotal role in the allergic sensitization of airways toward inhaled OVA (27, 28). Accordingly, we examined whether the observed allergic sensitization could be due to endotoxin-like effects of cigarette smoke. Therefore, we challenged TLR4-deficient mice with cigarette smoke and OVA simultaneously for 3 wk and measured the degree of eosinophilic inflammation. Remarkably, TLR4-deficient mice were clearly able to develop a Th2 response comparable to the levels observed in BALB/c control mice, as measured by BAL eosinophilia, BAL lymphocytes, BAL DCs, and serum OVA-specific IgE (Fig. 4A). In addition, histological evaluation of lung tissue showed peribronchial eosinophilic infiltrates in both TLR4-deficient and control mice exposed to cigarette smoke and OVA simultaneously (Fig. 4B).

To exclude the contribution of alternative TLRs in cigarette smoke-induced allergic airway sensitization, we performed experiments in strain-matched MyD88 gene-deficient animals. MyD88 is an essential adaptor molecule broadly shared among members of the TLR/IL-1R family. OVA/smoke-exposed MyD88 knockout mice did not show any significant reduction in eosinophilic airway inflammation, lymphocytic BAL infiltration, and airway DC recruitment (Fig. 4C). In contrast and consistent with earlier observations in murine models of cigarette smoke-induced lung inflam-

mation (37), neutrophil and macrophage recruitment into the airways in response to OVA/smoke inhalation was clearly impaired in the absence of MyD88.

Discussion

Concomitant inhalation of aerosolized OVA and cigarette smoke in mice induces a Th2-type airway inflammation that is absent after exposure to either agent alone. This aberrant immune response has high clinical relevance, as illustrated by the interaction between smoking habits and the development of asthma in predisposed subjects. The present study was designed to investigate the triggering of allergic airway inflammation by cigarette smoke from a pulmonary DC point of view. Key features of pulmonary DC biology are 1) recruitment to the Ag-exposed lung, 2) sampling of inhaled Ag and transport toward draining mediastinal LNs, and 3) maturation (i.e., increase in costimulatory molecule expression), which is necessary to generate Ag-specific effector T cells. Subsequently, we sought to gain mechanistic insight in the observed phenomenon: based on previous knowledge, we focused on potential endotoxin-like activity in cigarette smoke.

We first confirmed that combined smoke and OVA protein aerosol exposure induces a strong increase in DC numbers in the airway compartment, which is consistent with observations made previously in OVA/alum-sensitized, OVA aerosol-challenged mice (32, 38). Transport of Ags from the airways to the T cell areas of draining LNs represents an additional key step in the Ag-presenting function of airway DCs. In this study, we reveal that there is strongly amplified DC-mediated transport of

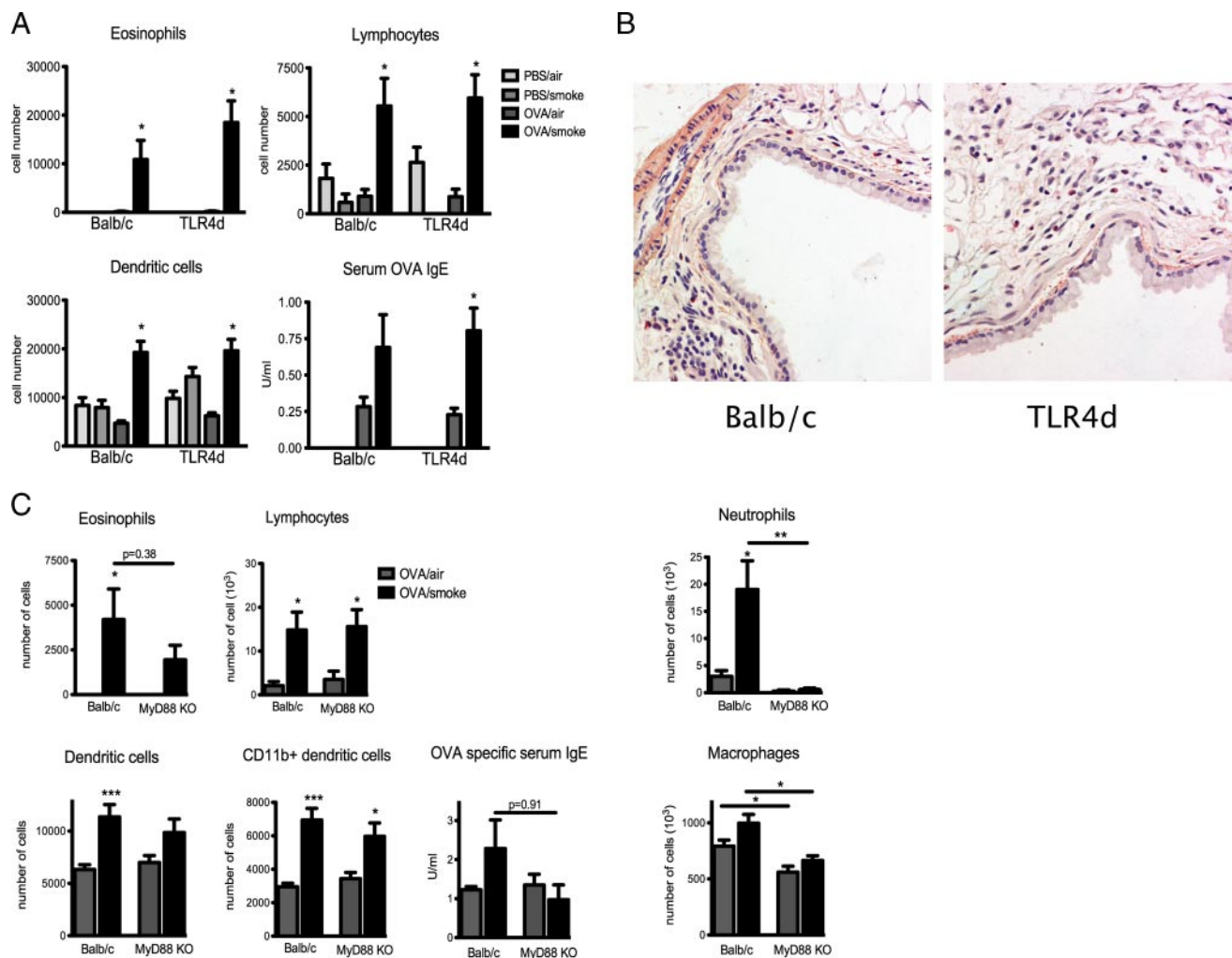


FIGURE 4. Allergic sensitization of TLR4-deficient and MyD88 gene-deficient mice exposed to cigarette smoke and OVA. *A*, Absolute numbers of eosinophils, lymphocytes, and DCs in the BAL fluid and concentration of OVA-specific IgE in serum of control mice (BALB/c) or TLR4-deficient mice (TLR4d) exposed to PBS or OVA combined with cigarette smoke or air for 3 wk. Eosinophils and lymphocytes are identified on cytospins stained with May-Grünwald-Giemsa, while DCs are identified by flow cytometry as low-autofluorescent CD11c^{high} cells. $n = 9$ mice/group. *B*, Histological evaluation of eosinophilic peribronchial infiltrates on Congo Red-stained lung tissue sections of BALB/c mice and TLR4-deficient mice counterstained with hematoxylin. *C*, Numbers of eosinophils, lymphocytes, neutrophils, DCs, and macrophages in the BAL fluid of MyD88 knockout mice or BALB/c control mice after 3 wk of OVA aerosol with (■) or without (□) cigarette smoke simultaneously. Eosinophils, lymphocytes, and neutrophils were identified on cytospins, whereas DCs (CD11c^{high} low autofluorescent cells), CD11b⁺ DCs (low-autofluorescent CD11b⁺CD11c^{high} cells) and macrophages (high-autofluorescent CD11c^{high} cells) were differentiated using FACS analysis. OVA-specific IgE is determined by ELISA. $n = 7$ mice/group. *, A significant difference ($p < 0.05$) between the OVA/air and OVA/smoke group, unless otherwise indicated.

inhaled protein Ag toward mediastinal LNs in the presence of coinhaled cigarette smoke. In accordance with this observation, we detected an up-regulation of the LN-homing chemokine receptor CCR7 on airway DCs. Migration of DCs to the LNs is enabled by CCR7 expression on the cell surface of DCs interacting with the chemokines CCL21 and CCL19 displayed on lung lymphatics and in T cell zones of LNs. An alternative explanation for the enhanced capture and transport of airway Ag by DCs could be the recently described alteration of airway epithelial tight junctions by mainstream cigarette smoke (39). The resulting increase in epithelial permeability could lead to enhanced penetration of airborne Ag and promotion of the activation of the subepithelial DC network (40). In addition to changes in DC trafficking to the LNs, perturbation of the epithelial homeostasis could also affect DCs in a way that can trigger Th2-oriented sensitization. GM-CSF for instance, a product of the airway epithelium that is increased in smoke-exposed airways (13, 17, 41), has been shown to induce allergic

sensitization to inhaled OVA by means of airway DC activation in the absence of any other adjuvant (42). Furthermore, allergic sensitization by cigarette smoking was recently found to be, at least partially, GM-CSF dependent (16). Thymic stromal lymphopoietin (TSLP) is another candidate cytokine which can be secreted by the respiratory epithelium and is a powerful activator of DCs leading to a Th2-polarized immune response in an OX40-OX40L-dependent manner (43). Interestingly, intranasal administration of cigarette smoke extract induced TSLP expression in the mouse lung and blocking TSLP activity inhibited OVA-specific Th2 responses and airway inflammation after concomitant cigarette smoke extract and OVA exposure (44). However, in our experiments, we could not detect significant modulation of GM-CSF or TSLP expression after either smoke or concomitant OVA/smoke inhalation, nor did we observe up-regulation of OX40L on pulmonary DC populations (data not shown).

The pattern of accessory or costimulatory molecule expression on the Ag-transporting DCs is a known determinant in the outcome

of immune responses. Our data show that DCs in the airway compartment of OVA/smoke-exposed lungs display an increased expression of accessory molecules previously known for their role in modulating allergic immune responses. In line with our earlier observations (34, 35), cigarette smoke inhalation induces up-regulation of CD86 (B7-2) on airway DCs. This costimulatory molecule has been clearly shown to be involved in the priming of Th2 responses and the subsequent development of allergic airway inflammation toward inhaled protein (32, 45), and could thus be a means through which cigarette smoke exerts its Th2-skewing properties in this model. The striking down-regulation of ICOSL on airway DCs of mice concomitantly exposed to cigarette smoke and OVA is also consistent with our previous observations in the OVA-alum-i.p./OVA-aerosol asthma model (32). Expression of ICOSL on DCs during initial priming of T cells drives the formation of regulatory T cells in lung draining LNs and has been involved in the establishment of respiratory tolerance (36). Withdrawal of this inhibitory signal might contribute to the preferential Th2 priming toward inhaled Ags. An intriguing observation is the up-regulation of PDL2, another member of the B7 family, on airway-derived DCs in the draining mediastinal LNs of mice with OVA/smoke-induced eosinophilic airway inflammation. This is reminiscent of the previously described increase in PDL2 on airway DCs of OVA-alum-sensitized/OVA aerosol-challenged allergic mice (46) and might thus be a characteristic feature of allergic immune responses. PDL2 costimulation during allergen priming and challenge was shown to exacerbate immune and inflammatory allergic responses in mice, supporting a Th2-potentiating function (47). It has also been proposed that Th2 cells specifically regulate PDL2 expression on DCs via reverse signaling (48, 49). The novel finding in our study, however, is a striking up-regulation of PDL2 on airway-derived LN DCs after cigarette smoke inhalation alone. Whether this creates a context prone to Th2-polarized sensitization toward coinhaled protein is a tempting hypothesis. Further investigations using, for example, blocking Abs or local delivery of small interfering RNA, may help to elucidate the role of DC-expressed PDL2 in the process of allergic sensitization.

Interestingly, cigarette smoke exposure resulted in a reduction of pDC numbers in the lungs and thoracic LNs. pDCs have been recently described for their capacity to maintain inhalational tolerance: selective depletion of pulmonary pDCs was able to spark Th2-polarized airway inflammation toward inhaled inert Ag in the absence of any adjuvant (33). Along with 1) the activation of conventional airway DCs, 2) the increase in DC-mediated transport of inhaled Ag toward LNs, and 3) specific modulation DC costimulatory molecule expression, the reduced number of pDCs in smoke-exposed lungs might thus establish a general climate of reduced inhalational tolerance.

In contrast to our current findings, a recent study by Robbins et al. (50) reported that cigarette smoke leads to a decrease in lung DC numbers, a decrease in DC activation markers, and no change in migration toward mediastinal LNs. These findings are likely attributable to fundamental methodological differences. The most important element (not obvious in the above-mentioned study) is the exposure system used, i.e., nose only vs whole body, as this leads to large differences in dose intensity as reflected by carboxy-hemoglobin levels. The protocol we used involves more frequent exposures and results in carboxyhemoglobin levels comparable to those measured in human smokers (51, 52). In addition, we report surface expression of costimulatory molecules on DCs as shifts in mean fluorescence intensity (MFI), which is more accurate than percent positive cells for these types of markers. Finally, in our study, we aim to discriminate effects on lung parenchymal DCs obtained by enzymatic digestion vs airway DCs harvested by BAL.

This allows us to detect the confinement of smoke effects (e.g., the up-regulation of accessory molecules and CCR7) to the DCs in the airway compartment.

We sought to gain mechanistic insight in the Th2-skewing adjuvant properties of cigarette smoke coinhaled with inert protein. We hypothesized that this effect would rely on the presence of endotoxin-like activity within the smoke itself. Indeed, both mainstream and side-stream cigarette smoke are known to contain bioactive endotoxin (30). It was already shown that, whereas endotoxin-free OVA induces inhalational tolerance, coinhalation of OVA and low-dose endotoxin triggers OVA-specific allergic airway inflammation (27). This phenomenon, along with the activation and enhanced migration of airway DCs, appeared to be TLR4 dependent (28). TLR4 also appears to be a determining factor in the initiation of allergic responses against inhaled house dust mite allergen (53, 54). Furthermore, our group and others previously revealed that acute cigarette smoke exposure induces airway DC maturation in a TLR4-dependent fashion (34, 37). Therefore, we were surprised to observe unhampered development of allergic airway inflammation in TLR4-deficient mice exposed to cigarette smoke and OVA. Any endotoxin potentially contaminating the cigarette smoke and/or the OVA used in these studies would have its proallergic effects greatly diminished in the absence of functional TLR4. Cigarette smoke exposure could also activate alternative TLRs, such as TLR2 which can bind heat shock protein 70, an endogenous danger signal known to be released in smoke-exposed airways (37). However, smoke-induced allergic sensitization was unhampered in Myd88 gene-deficient animals, thereby ruling out the contribution of TLR2 and several other members of the TLR/IL-1R family of innate immune sensors. This is in contrast to airway neutrophilia that disappeared completely in our model (consistent with Ref. 37) and the MyD88 dependency of allergic responses observed after house dust mite challenge, intranasal challenge of OVA with low-dose LPS, or in the presence of bacterial infection (29, 55, 56).

The fact that in our experiments the development of allergic inflammation is unaffected by TLR4/MyD88 deficiency suggests immunological adjuvant mechanisms of a different kind. The cellular stress inflicted by cigarette smoke in the airways, for instance, could lead to the release of compounds that trigger TLR/MyD88-independent immunogenic pathways. One of these endogenous compounds could be ATP, which was recently reported to trigger purinergic receptors on the airway DC network, leading to a DC-dependent allergic sensitization toward inhaled inert protein (57). Furthermore, alum, a prototypical Th2-skewing adjuvant, was shown to exert its adjuvant effect in a TLR4-independent way and to activate the NLRP3 inflammasome pathway either directly or through the release of uric acid from the local tissue microenvironment (58, 59). It is worth noting that aluminum has been detected at high concentrations in cigarette smoke, potentially precipitating as alum-like hydroxy salts in the epithelial lining fluid overlying the airway DC network (60). In support of this idea, MyD88 signaling is redundant for alum to act as an adjuvant for humoral adaptive responses (61). Hence, cytotoxic compounds in cigarette smoke could act in a TLR/MyD88-independent manner by activating airway DCs either directly or by means of endogenous danger signals released by stressed airway cells (reviewed in Ref. 62). Whether this could result in a deviation of immune homeostasis toward an aberrant Th2-driven inflammation is a compelling working hypothesis for further investigation.

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Disclosures

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