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Sirtuin 1 Promotes Th2 Responses and Airway Allergy by Repressing Peroxisome Proliferator-Activated Receptor- γ Activity in Dendritic Cells

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Sirtuins are a unique class of NAD⁺-dependent deacetylases that regulate diverse biological functions such as aging, metabolism, and stress resistance. Recently, it has been shown that sirtuins may have anti-inflammatory activities by inhibiting proinflammatory transcription factors such as NF- κ B. In contrast, we report in this study that pharmacological inhibition of sirtuins dampens adaptive Th2 responses and subsequent allergic inflammation by interfering with lung dendritic cell (DC) function in a mouse model of airway allergy. Using genetic engineering, we demonstrate that sirtuin 1 represses the activity of the nuclear receptor peroxisome proliferator-activated receptor- γ in DCs, thereby favoring their maturation toward a pro-Th2 phenotype. This study reveals a previously unappreciated function of sirtuin 1 in the regulation of DC function and Th2 responses, thus shedding new light on our current knowledge on the regulation of inflammatory processes by sirtuins. *The Journal of Immunology*, 2011, 187: 000–000.

CD4⁺ Th cells play a central role in the orchestration of adaptive immune responses and are important for protective immunity to many infectious agents. In response

to Ag stimulation, naive Th cells may differentiate into at least four types of effector Th cells, namely Th1, Th2, Th17, and inducible regulatory T cells, which differ from each other in their cytokine secretion profile and function (1). Th2 cells, which produce IL-4, IL-5, and IL-13, are indispensable for host immunity to extracellular parasites, such as helminths (2). However, it has also been demonstrated that inappropriate Th2 responses to innocuous environmental Ags are responsible for the development of allergic diseases (3).

Dendritic cells (DCs) are professional APCs required for efficient stimulation of naive Th cells and initiation of primary immune responses (4). They are also needed for optimal restimulation of effector Th cells. Although Th1 induction by DCs is well documented, and rapid progress is being made on Th17 stimulation, the molecular mechanisms by which DCs direct Th2 responses remain more elusive (3). Yet, a better understanding of these mechanisms could help in the design of new therapeutic approaches for allergic disorders such as atopic asthma, a chronic inflammatory condition of the airways that is increasing in severity and prevalence in Western countries (5, 6).

The mammalian sirtuin family of proteins comprises seven NAD⁺-dependent protein deacetylases (Sirt1–7) homologous to the yeast Sir2 protein (7–9). The seven members of the family share a conserved catalytic domain but show differences in their enzymatic activity (7–9). Although Sirt1, Sirt2, Sirt3, and Sirt5 deacetylate histone and nonhistone protein substrates, Sirt4 and Sirt6 mainly act as mono-ADP-ribosyl transferases. The enzymatic activity of Sirt7 has not yet been characterized.

The sirtuins have been implicated in a variety of essential biological functions including cell cycle progression, apoptosis, cellular senescence, stress response, neuronal protection, adaptation to caloric restriction, metabolism, tumorigenesis, and transcription of rRNA genes (8–13). In vitro studies have shown that Sirt1 may also limit the inflammatory process by inhibiting NF- κ B and AP-1 (14–18), two transcription factors crucially involved in the expression of proinflammatory cytokines such as TNF- α .

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Abbreviations used in this article: AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; BLN, bronchial lymph node; BMDC, bone marrow-derived dendritic cell; cKO, conditional knockout; COPD, chronic obstructive pulmonary disease; DC, dendritic cell; FOXO, forkhead box class O; HIF, hypoxia-inducible factor; i.t., intratracheal; MHC II, MHC class II; PAS, periodic acid-Schiff; PGK, phosphoglycerate kinase; PPAR, peroxisome proliferator-activated receptor; WT, wild-type.

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Consistent with these findings, lung cells from patients with chronic obstructive pulmonary disease (COPD) and from rats exposed to cigarette smoke display reduced expression of Sirt1 associated with increased NF- κ B activity and matrix metalloproteinase-9 expression as compared with lung cells from healthy controls (15, 17). Another argument supporting an anti-inflammatory role for Sirt1 is that transgenic *Sirt1* mice under a high-fat diet show lower lipid-induced NF- κ B-dependent inflammation than their wild-type (WT) counterparts (19). Moreover, many independent studies have shown that deletion of Sirt1 leads to metabolic diseases associated with inflammatory responses (20–23). Sirt6, like Sirt1, is able to attenuate NF- κ B-dependent gene expression (24). However, a recent study revealed that Sirt6 also has the surprising capacity to upregulate the expression of TNF- α by acting at a posttranscriptional level (25), indicating that regulation of the inflammatory response by sirtuins is subtler than previously thought. Consistent with this observation, Lee and collaborators (26, 27) reported that treatment with sirtinol, an inhibitor of Sirt1 and Sirt2, reduces airway inflammation and hyperreactivity in a mouse model of atopic asthma, through mechanisms that yet remain to be formally identified (28).

In this study, we show that mice treated with pharmacological sirtuin inhibitors (i.e., sirtinol or cambinol) are impaired in their ability to mount adaptive Th2 responses and therefore allergic airway inflammation, due to decreased maturation, migration, and pro-Th2 accessory activity of DCs. Using conditional models of genetic depletion, we further demonstrate that differentiation of DCs toward a pro-Th2 phenotype requires inhibition of peroxisome proliferator-activated receptor (PPAR)- γ by Sirt1. This study reveals a previously unappreciated role for Sirt1 in modulating DC function and Th2 responses, thus shedding new light on our current knowledge of the regulation of inflammatory processes by sirtuins.

Materials and Methods

Mice

Female BALB/c and C57BL/6 mice were purchased from Harlan Laboratories. OVA-specific, MHC class II (MHC II)-restricted, TCR-transgenic DO11.10 (H-2^d; BALB/c background) and OT-II (H-2^b; C57BL/6 background) mice, CD11c-CRE transgenic mice (C57BL/6 background), as well as *Ppar- γ* -floxed (*Ppar- γ ^{fl/fl}*) mice (C57BL/6 background) and *Sirt1^{fl/fl}* mice (C57BL/6 background) were from The Jackson Laboratory. *Sirt1^{fl/fl}* mice were crossed with CD11c-CRE transgenic mice for generating *Sirt1* conditional knockout (cKO) mice. All mice were housed under specific pathogen-free conditions and used at 8–12 wk of age. All experiments were conducted with Institutional Animal Care and Use Committee approval.

Reagents and Abs

Cambinol and sirtinol were purchased from Calbiochem and Alexis Biochemicals, respectively. OVA grade III and grade V and methacholine were purchased from Sigma-Aldrich. Inject Alum was from Pierce. FITC-OVA was from Molecular Probes Invitrogen. Recombinant murine GM-CSF was produced in-house. PE-conjugated anti-F4/80 (BM8), PE-Cy7-conjugated anti-CCR7 (4B12), allophycocyanin-eFluor780 anti-CD11c (N418), eFluor450-anti-CD4 (RM4-5) and anti-CD19 (1D3) were from eBioscience. Biotinylated anti-MHC II I-E^k (17-3-3) and I-A^b (AF6-120.1), PE-anti-CD3e (145-2C11), and FITC-anti-CD8 α (53-6.7) were from BD Biosciences. FITC-conjugated anti-CD40 (3/23), anti-CD80 (RM80), and anti-CD86 (PO3) were from Serotec. 2.4G2 Fc receptor Abs were produced in-house. Allophycocyanin- and FITC-streptavidin were from eBioscience. Isotype controls were from the same manufacturer as their corresponding Abs.

Induction of allergic airway disease and treatment of mice with cambinol and sirtinol

BALB/c mice were sensitized on days 0 and 14 by i.p. injection of 10 μ g OVA (grade V) dissolved in 100 μ l PBS and mixed with 100 μ l Inject Alum (Pierce). Sham-immunized mice received 100 μ l PBS mixed with 100 μ l Inject Alum (Pierce). On days 28–32, mice were challenged by

exposure to an aerosol of 1% OVA (w/v; grade III) in PBS for 1 h. Twenty-four hours after the last challenge (day 33), airway hyperresponsiveness (AHR) was measured, the mice were killed, and the severity of allergic airway inflammation was assessed.

To assess the effects of specific inhibition of sirtuins on pulmonary allergy, cambinol or sirtinol were injected i.p. to OVA-sensitized mice 1 h prior to each OVA inhalation. Cambinol was administered at the dose of 100 mg/kg in 500 μ l 10%/10% ethanol/Cremophore solution (29). Sirtinol was given at the dose of 50 mg/kg in 50 μ l 100% DMSO solution. Control mice received vehicle alone.

Bronchoalveolar lavage and cytology

The trachea was catheterized, and the lungs were lavaged with 1 ml ice-cold Mg- and Ca-free PBS containing 0.6 mM EDTA. Cell density in bronchoalveolar lavage fluid (BALF) was assessed by the use of a hemocytometer. Cell differentials were performed on cytospin preparations stained with Diff-Quick (Dade Behring).

Lung histology

Lungs were fixed in 10% formalin, paraffin embedded, cut in 5- μ m sections, and stained with H&E. Intracytoplasmic and luminal mucin was assessed by periodic acid-Schiff (PAS) stains. Mucus production was quantified as the percentage of PAS-stained goblet cells per total epithelial cells in randomly selected bronchi. Seven randomly selected sections were analyzed per murine lung.

Restimulation of bronchial lymph node cells

Bronchial lymph node (BLN) cells (2×10^5 cells in a 96-well plate) were cultured in Click's medium supplemented with 0.5% heat-inactivated mouse serum and additives, with or without OVA (50 μ g/ml, grade V; Sigma-Aldrich). The proliferation was measured as [³H]thymidine incorporation during the last 16 h of a 3-d culture. Culture supernatants were assayed for IL-4, IL-5, IL-13, and IFN- γ by ELISA (Biosource International).

Measurement of AHR

Mice were anesthetized by i.p. injection (200 μ l) of a mixture of ketamine (10 mg/ml; Merial) and xylazine (1 mg/ml; VMD). A tracheotomy was performed by insertion of a 20-gauge polyethylene catheter into the trachea. A ligature was performed around the catheter to avoid leaks and disconnections. Mice were ventilated with a flexiVent small animal ventilator (SCIREQ) at a frequency of 450 breaths per minute and a tidal volume of 10 ml/kg. A positive end expiratory pressure was set at 2 h Pa. Measurement started after 2 min of mechanical ventilation. A sinusoidal 1-Hz oscillation was then applied to the tracheal tube and allowed a calculation of dynamic resistance, elastance, and compliance of the airway by multiple linear regressions. Following measurement of baseline lung function, mice were exposed to a saline aerosol (PBS) followed by aerosols containing increasing doses (3, 6, 9, and 12 g/l) of methacholine. Aerosols were generated by the mean of an ultrasonic nebulizer (SCIREQ) and delivered to the inspiratory line of the flexiVent (SCIREQ) using a bias flow of medical air following the manufacturer's instructions. Each aerosol was delivered for 10 s, and periods of measurement as described above were made at 1-min intervals following each aerosol. Dynamic resistance was the main parameter measured during the challenge (30).

Flow cytometry

Staining reactions were performed at 4°C. Cells were incubated with 2.4G2 FcR Abs to reduce nonspecific binding. Analyses were performed on an FACSCanto II flow cytometer (BD Biosciences).

Cell isolation and culture

To obtain single lung cell suspensions, lungs were perfused with 20 ml PBS through the right ventricle, cut into small pieces, and digested for 1 h at 37°C in 1 mg/ml collagenase A (Roche) and 0.05 mg/ml DNaseI (Roche) in HBSS. Lung DCs were sorted by flow cytometry (FACS Aria; BD Biosciences) based on their differential expression of CD11c and MHC II. Alveolar macrophages, which are also positive for CD11c and MHC II, were excluded based on their autofluorescence and high expression of F4/80 (31). DO11.10 and OT-II CD4⁺ T cells were isolated from the spleen using magnetic bead purification (CD4⁺ T Cell Isolation Kit 130-090-860; Miltenyi Biotec). Isolated cells were either directly used for experiments or cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 50 μ M 2-ME, 50 μ g/ml streptomycin, and 50 IU/ml penicillin (all from Life Technologies-Invitrogen).

RNA purification, cDNA synthesis, and RT-PCR

Total RNA was extracted from the cells using TRIzol Reagent (Invitrogen). Poly(A) RNA was primed with oligo(dT) (Roche) and reverse transcribed with AMV reverse transcriptase (Roche) for 1 h at 42°C. cDNA products were amplified by PCR using primers specific for mouse *Sirt1* (5' primer, 5'-CAG ACC CTC AAG CCA TGT TT-3'; 3' primer, 5'-ACA CAG AGA CGG CTG GAA CT-3'), *Sirt2* (5' primer, 5'-TGG AGA GGC AGA GAT GGA CT-3'; 3' primer, 5'-GTC ACT CCT TCG AGG GTC AG-3'), *Sirt3* (5' primer, 5'-TAC AGG CCC AAT GTC ACT CA-3'; 3' primer, 5'-GGA TCC CAG ATG CTC TCT CA-3'), *Sirt4* (5' primer, 5'-CAT CCA GCA CAT TGA TTT CG-3'; 3' primer, 5'-GTT GGG TTG GTG AGA GGA GA-3'), *Sirt5* (5' primer, 5'-CAT CAC CCA GAA CAT TGA CG-3'; 3' primer, 5'-CAG TGC CAC AGG TAC AC-3'), *Sirt6* (5' primer, 5'-AGC TGA GAG ACA CCA TTC TGG-3'; 3' primer, 5'-CCA GGG TGA CAG ACA GGT CT-3'), *Sirt7* (5' primer, 5'-AGC TTC GGG ATA CCA TTG TG-3'; 3' primer, 5'-CAG GAT TGT GTC TGC TTT GC-3'), and *Ppar-γ* (5' primer, 5'-GTC ACG TTC TGA CAG GAC TGT GTG AC-3'; 3' primer, 5'-TAT CAC TGG AGA TCT CCG CCA ACA GC-3'). All primers were purchased from Eurogentec. A 50 μl PCR reaction was set up containing 5 μl cDNA, 10 mM Tris-HCl, 25 pmol each primer, 1.5 mM MgCl₂, 0.2 mM 2'-deoxynucleoside 5'-triphosphate, and 2.5 U AmpliTaq DNA polymerase (PerkinElmer). Amplification consisted of 30 (sirtuins) or 40 (PPAR-γ) cycles of denaturation at 95°C for 20 s, annealing at 55°C (sirtuins) or 60°C (PPAR-γ) for 30 s, and extension at 72°C for 1 min. Amplification products were electrophoresed on 1.2% agarose gels and visualized by ethidium bromide staining.

Evaluation of lung DC migration

In vivo migration of lung DCs was assessed by injecting 800 μg FITC-labeled OVA in the trachea of mice that had been treated with vehicle, cambinol, or sirtinol 18 and 3 h before. Twenty-four hours later, BLNs were collected, and single-cell suspensions were analyzed by flow cytometry for the presence of FITC⁺ DCs (CD11c⁺MHC II⁺F4/80⁺ cells). Dead cells were excluded using DAPI (Molecular Probes Invitrogen). Cambinol was given i.p. 18 and 3 h before intratracheal (i.t.) injection of FITC-OVA at the dose of 100 mg/kg in 500 μl 10%/10% ethanol/cremophore solution. Sirtinol was administered i.p. at the same time points at the dose of 50 mg/kg in 50 μl 100% DMSO solution. Control mice received the vehicle alone.

Apoptosis assays

Cells were assayed for apoptosis using dual-color annexin V-FITC/propidium iodide staining (Annexin V-FLUOS staining kit; Roche Applied Sciences) and flow cytometry analyses.

Generation of bone marrow-derived DCs

Bone marrow cells were collected from naive mice, depleted of RBCs using ammonium chloride lysis buffer, and grown for 8 d in DMEM supplemented with 10% FCS, 1% L-glutamine, 1% sodium pyruvate, 0.1% 2-ME, 50 μg/ml streptomycin, 50 IU/ml penicillin, and 20 ng/ml recombinant murine granulocyte/M-CSF (32). At day 8, >90% of bone marrow cells were positive for the DC marker CD11c and considered as bone marrow-derived DCs (BMDCs). In all experiments, BMDCs were stimulated at day 8.

Stimulation of OVA-specific CD4⁺ T cells by DCs

FACS-sorted lung DCs (CD11c⁺MHC II⁺F4/80⁺ cells; 10⁶ cells/ml) or BMDCs from naive BALB/c or C57BL/6 mice were stimulated for 16 h with 125 μg/ml OVA in the presence of vehicle alone (0.1% DMSO), 50 μM cambinol, or 25 μM sirtinol. DCs were then extensively washed, irradiated, and cocultured for 72 h with freshly isolated DO11.10 or OT-II CD4⁺ T cells (1 × 10⁴ DCs were used to stimulate 2 × 10⁵ DO11.10 or OT-II T cells). Unpulsed, vehicle-treated DCs were used as controls. The proliferation of DO11.10 or OT-II T cells was measured as [³H]thymidine incorporation during the last 16 h of the 72-h culture. Culture supernatants were assayed for IL-4, IL-5, and IL-13 by ELISA (Biosource International).

Th2 sensitization induced by i.t. administration of OVA-pulsed BMDCs

At day 8 of culture, BMDCs were stimulated with 125 μg/ml OVA in the presence of vehicle, cambinol, or sirtinol. At day 9, cells were collected, washed, and resuspended in PBS. A total of 10⁶ cells were then injected i.t. to anesthetized naive recipients. Unpulsed, vehicle-treated BMDCs were used as controls. Ten days after i.t. immunization, mice were challenged with OVA (1% w/v in PBS; grade III) aerosol during a daily 30 min

challenge on 5 consecutive d. Twenty-four hours after the last challenge, the mice were killed, and airway allergy was evaluated as described above.

Nuclear protein extraction

Nuclear protein extracts were prepared as previously described (33). Cytoplasmic buffer (pH 7.9) contained 10 mM HEPES, 10 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 0.2% (v/v) Nonidet P-40, 1.6 mg/ml protease inhibitors (Complete; Roche), and 3 mM serine protease inhibitor diisopropyl fluorophosphate (Sigma-Aldrich). Pelleted nuclei were resuspended in 20 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 0.2 mM EDTA, 0.63 M NaCl, 25% (v/v) glycerol, 1.6 mg/ml protease inhibitors, and 3 mM DFP (nuclear buffer), incubated for 20 min at 4°C, and centrifuged for 30 min at 14,000 rpm. Protein amounts were quantified with the Micro BCA protein assay reagent kit (Pierce).

EMSAs

Binding reactions were performed for 30 min at room temperature with 5 μg nuclear proteins in 20 mM HEPES (pH 7.9), 10 mM KCl, 0.2 mM EDTA, 20% (v/v) glycerol, 1% (w/v) acetylated BSA, 3 μg poly(deoxyinosinic-deoxycytidylic) acid (Amersham Pharmacia Biotech), 1 mM DTT, 1 mM PMSF, and 100,000 cpm ³²P-labeled double-stranded oligonucleotide probes. Probes were prepared by annealing the appropriate single-stranded oligonucleotides (Eurogentec) at 65°C for 10 min in 10 mM Tris, 1 mM EDTA, and 10 mM NaCl, followed by slow cooling to room temperature. The probes were then labeled by end-filling with the Klenow fragment of *Escherichia coli* DNA polymerase I (Roche), with (α-[³²P])–2'-deoxyadenosine triphosphate and (α-[³²P])–2'-deoxycytidine triphosphate (Dupont-New England Nuclear Life Science Products). Labeled probes were purified by spin chromatography on Sephadex G-25 columns (Roche). DNA-protein complexes were separated from unbound probe on 4% native polyacrylamide gels at 150 V in 0.25 M Tris, 0.25 M sodium borate, and 0.5 mM EDTA (pH 8). Gels were vacuum-dried and exposed to Fuji x-ray film (Fujifilm) at –80°C for 12 h. To confirm specificity, competition assays were performed with a 50-fold excess of unlabeled WT probes and with mutated probes. The sequences of the oligonucleotides used in this work were as follows: WT PPAR-γ probe, 5'-TTG GCA AAA CTA GGT CAA AGG TCA CA-3' (forward) and 5'-TTG GTG TGA CCT TTG ACC TAG TTT TG-3' (reverse); and mutated PPAR-γ probe, 5'-TTG GCA AAA CTA GCA CAA AGC ACA CA-3' (forward) and 5'-TTG GTG TGT GCT TTG TGC TAG TTT TG-3' (reverse) (34).

Construction of lentiviral vectors and production of lentiviruses

A lentiviral construct encoding the Cre recombinase under the phosphoglycerate kinase (PGK) promoter was generated by subcloning the PGK promoter from pPGKpuro and the Cre recombinase from pBOB-CAGGS-iCre-SD into the pCSC-SP-PW lentiviral vector [kind gifts from Dr. Inder M. Verma, Salk Institute for Biological Studies, La Jolla, CA (35)]. Lentiviral particles were produced using a third-generation lentiviral packaging system, concentrated, and titrated as previously described (35).

Transduction of BMDCs

The BMDC culture medium was replaced at day 4 by medium containing recombinant viral particles at a multiplicity of infection of 15 and 5 μg/ml protamine sulfate (36). Six hours later, infecting medium was replaced by fresh culture medium. At day 8, the cells were treated or not with cambinol or sirtinol and pulsed or not with OVA. They were collected and used at day 9.

Statistical analysis

Data are presented as means ± SDs. Normality of the data distributions was assessed using Anderson-Darling tests. The differences between values were subsequently estimated using an ANOVA test followed by a Fisher's protected least SD test. A value <0.05 was considered significant. All of the experiments were repeated at least three times.

Results

Pharmacological inhibition of sirtuins during allergen challenge suppresses all the characteristic features of airway allergy

Recent evidence indicates that inhibition of sirtuins using the broad-spectrum inhibitor sirtinol decreases eosinophilic airway inflammation in a mouse model of allergic asthma (28). To obtain

more certainty about the specificity of these effects, we repeated these experiments using sirtinol (Supplemental Fig. 1), but also cambinol (Fig. 1), another pharmacological inhibitor of sirtuins (29). Sirtuin inhibition was achieved by i.p. administration of the inhibitors to mice previously sensitized with OVA 1 h before each repeated airway challenge with aerosolized OVA. OVA-sensitized mice exposed to aerosolized OVA and treated with sirtinol or cambinol are hereafter referred to as OVA/sirtinol-OVA and OVA/cambinol-OVA mice, respectively. Control OVA-sensitized mice exposed to aerosolized OVA that were treated with vehicle alone are called OVA/vehicle-OVA mice. Sham-immunized (PBS in Imject Alum) mice that were injected with vehicle during OVA challenge served as negative controls (PBS/vehicle-OVA mice).

As expected in this model, OVA/vehicle-OVA mice, but not PBS/vehicle-OVA mice, developed peribronchial and perivascular lung tissue eosinophilia and lymphocytosis, accompanied by goblet cell hyperplasia and increased mucus production (Fig. 1A, 1B). These modifications were congruently reflected in the increased total and eosinophil counts measured in the BALF of OVA/vehicle-OVA mice compared with PBS/vehicle-OVA animals (Fig. 1C). All of these inflammatory signs were significantly reduced when sensitized mice were treated with cambinol or sirtinol during OVA challenge (Fig. 1A–C, Supplemental Fig. 1A–C). Moreover, Th2 cytokine production and T cell proliferation in BLNs were significantly attenuated in OVA/sirtinol-OVA and OVA/cambinol-OVA mice compared with OVA/vehicle-OVA counterparts (Fig.

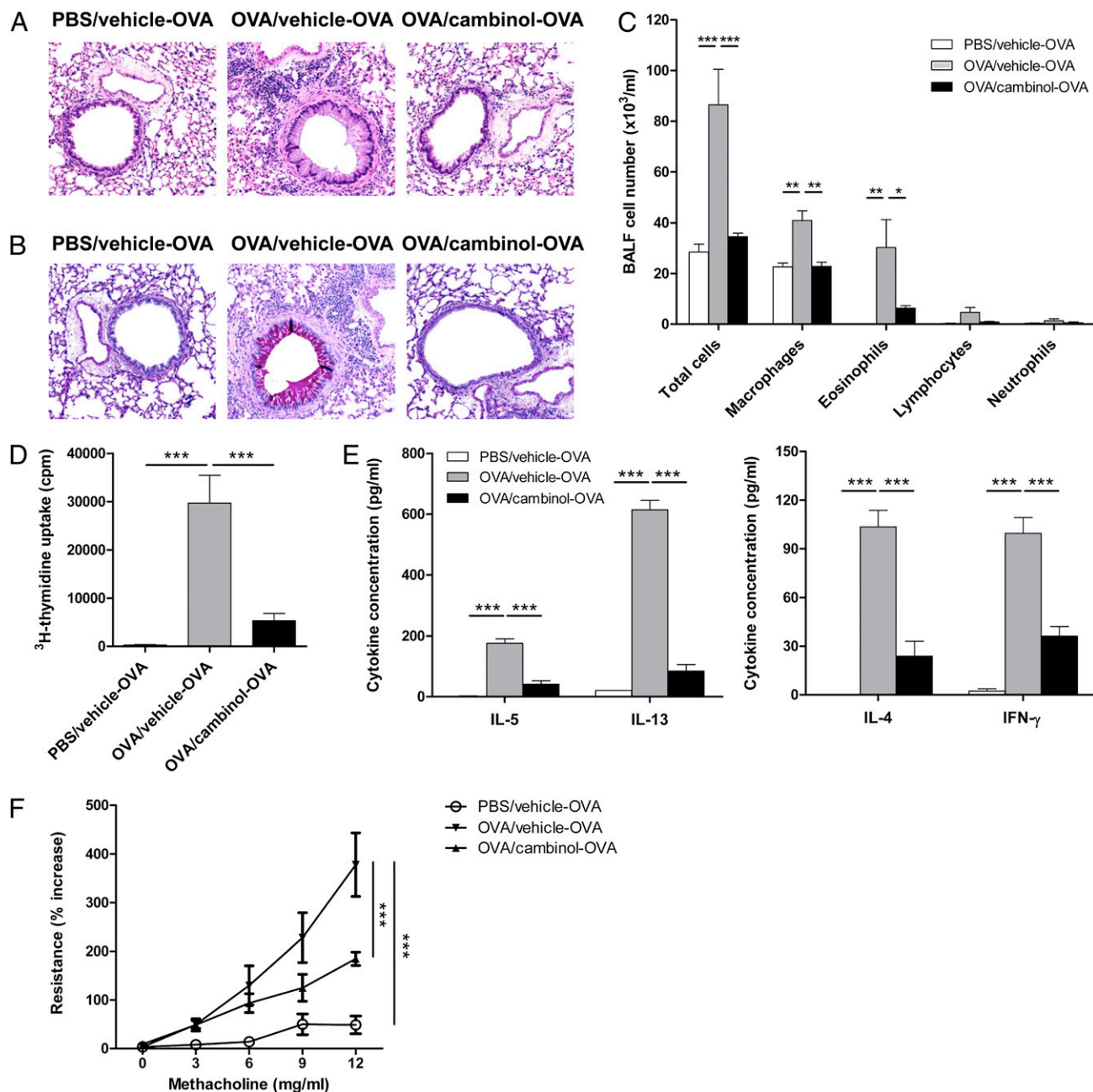


FIGURE 1. Treatment with cambinol markedly attenuates airway allergy. H&E (A) or PAS-stained (B) lung sections of PBS/vehicle-OVA, OVA/vehicle-OVA, and OVA/cambinol-OVA BALB/c mice (original magnification $\times 100$). C, Total and differential cell counts in the BALF. D, T cell proliferation estimated by measuring [^3H]thymidine uptake during the last 16 h of a 3-d *in vitro* culture of BLN cells with OVA. E, ELISA for cytokines in the BLN cell-culture supernatants. F, AHR to increasing doses of methacholine assessed by invasive measurement of dynamic resistance. Data shown are representative of three independent experiments using six to eight mice in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

1D, 1E, Supplemental Fig. 1D, 1E). Finally, AHR to nonspecific stimuli, a cardinal feature of allergic asthma, was significantly decreased in OVA/cambinol-OVA and OVA/sirtinol-OVA mice compared with OVA/vehicle-OVA animals (Fig. 1F, Supplemental Fig. 1F).

Together, these results support that pharmacological inhibition of sirtuins during allergen provocation may suppress the cardinal features of airway allergy and therefore support a crucial role for these proteins in the development of allergic airway inflammation and AHR.

Treatment with cambinol and sirtinol alters the ability of lung DCs to induce Th2 responses

Th2 responses were significantly downregulated in OVA/sirtinol-OVA and OVA/cambinol-OVA mice, raising the possibility that they were not optimally activated. Lung DCs are powerful APCs

that have been found to be necessary and sufficient for Th2 cell stimulation during ongoing allergic airway inflammation (37). We therefore hypothesized that cambinol and sirtinol could exert anti-inflammatory effects by interfering with the function of these cells. To support our assumption, we first assessed by RT-PCR the expression of sirtuins in lung DCs. As shown in Fig. 2A, lung DCs expressed Sirt1, Sirt2, and Sirt7 mRNA, whereas the transcripts of Sirt3, Sirt4, Sirt5, and Sirt6 were only barely detectable.

Migration of lung DCs to the BLNs following allergen challenge is required for efficient activation of Th2 cells and, therefore, for initiation and maintenance of airway allergic inflammation (37, 38). We thus examined whether treatment with sirtuin inhibitors affected the migratory properties of lung DCs. First, the total number of DCs in draining BLNs was counted in PBS/vehicle-OVA, OVA/vehicle-OVA, OVA/cambinol-OVA, and OVA/sirtinol-OVA mice 24 h following the last challenge with aerosolized

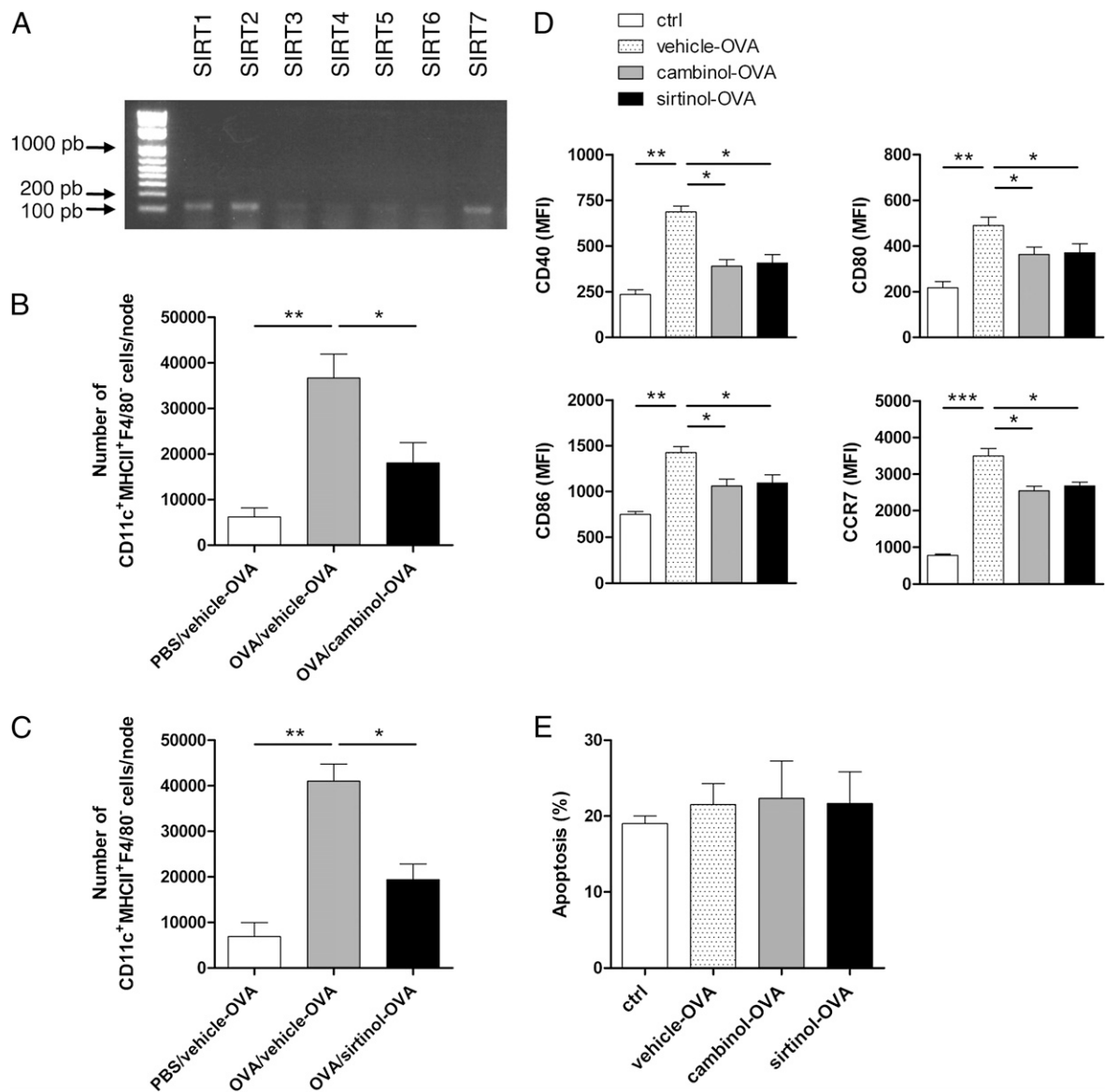


FIGURE 2. Lung DCs express Sirt1, Sirt2, and Sirt7, and sirtuin inhibition impairs DC maturation and migration. *A*, RT-PCR analysis of the expression of sirtuins (Sirt1–7) in CD11c⁺MHC II⁺F4/80⁻ lung DCs. Flow cytometric analysis of the number of CD11c⁺MHC II⁺F4/80⁻ DCs in the BLNs of PBS/vehicle-OVA, OVA/vehicle-OVA, OVA/cambinol-OVA (*B*), and OVA/sirtinol-OVA BALB/c mice (*C*). *D* and *E*, FACS-sorted lung DCs (CD11c⁺MHC II⁺F4/80⁻ cells) from naive BALB/c mice were stimulated with OVA in the presence of vehicle, cambinol, or sirtinol. *D*, Flow cytometric assessment of CD40, CD80, CD86, and CCR7 expression on lung DCs. We used freshly isolated lung DCs as controls (ctrl). *E*, Percentage of apoptotic DCs assayed using dual-color annexin V-FITC/propidium iodide staining and flow cytometry analyses. Data shown are representative of three independent experiments. *n* = 4. **p* < 0.05, ***p* < 0.01, ****p* < 0.001. MFI, mean fluorescence intensity.

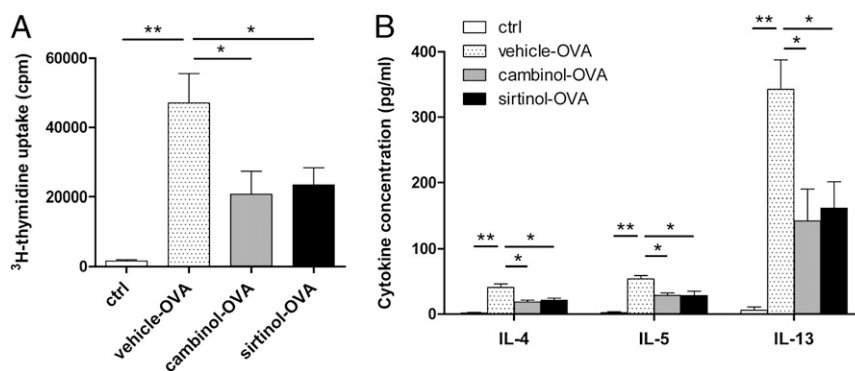


FIGURE 3. Sirtuin inhibition compromises the ability of DCs to stimulate Ag-specific T cells. *A* and *B*, FACS-sorted lung DCs ($\text{CD11c}^+\text{MHC II}^+\text{F4/80}^-$ cells) from naive BALB/c mice were stimulated with OVA in the presence of vehicle, cambinol, or sirtinol for 8 h. Lung DCs were then extensively washed, irradiated, and cocultured for 3 d with OVA-specific DO11.10 CD4^+ T cells. Unpulsed, vehicle-treated lung DCs were used as controls (ctrl). *A*, T cell proliferation evaluated by measuring [^3H]thymidine uptake during the last 16 h of culture. *B*, ELISA for cytokines in the culture supernatants. Data shown are representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$.

OVA. As previously described (38), OVA/vehicle-OVA mice displayed a significant increase in the number of DCs in BLNs compared with PBS/vehicle-OVA counterparts (Fig. 2*B*, 2*C*). In-

terestingly, the influx of DCs in BLNs was strongly reduced when sensitized mice were treated with either cambinol or sirtinol prior to each OVA challenge (Fig. 2*B*, 2*C*). Of note, the number of

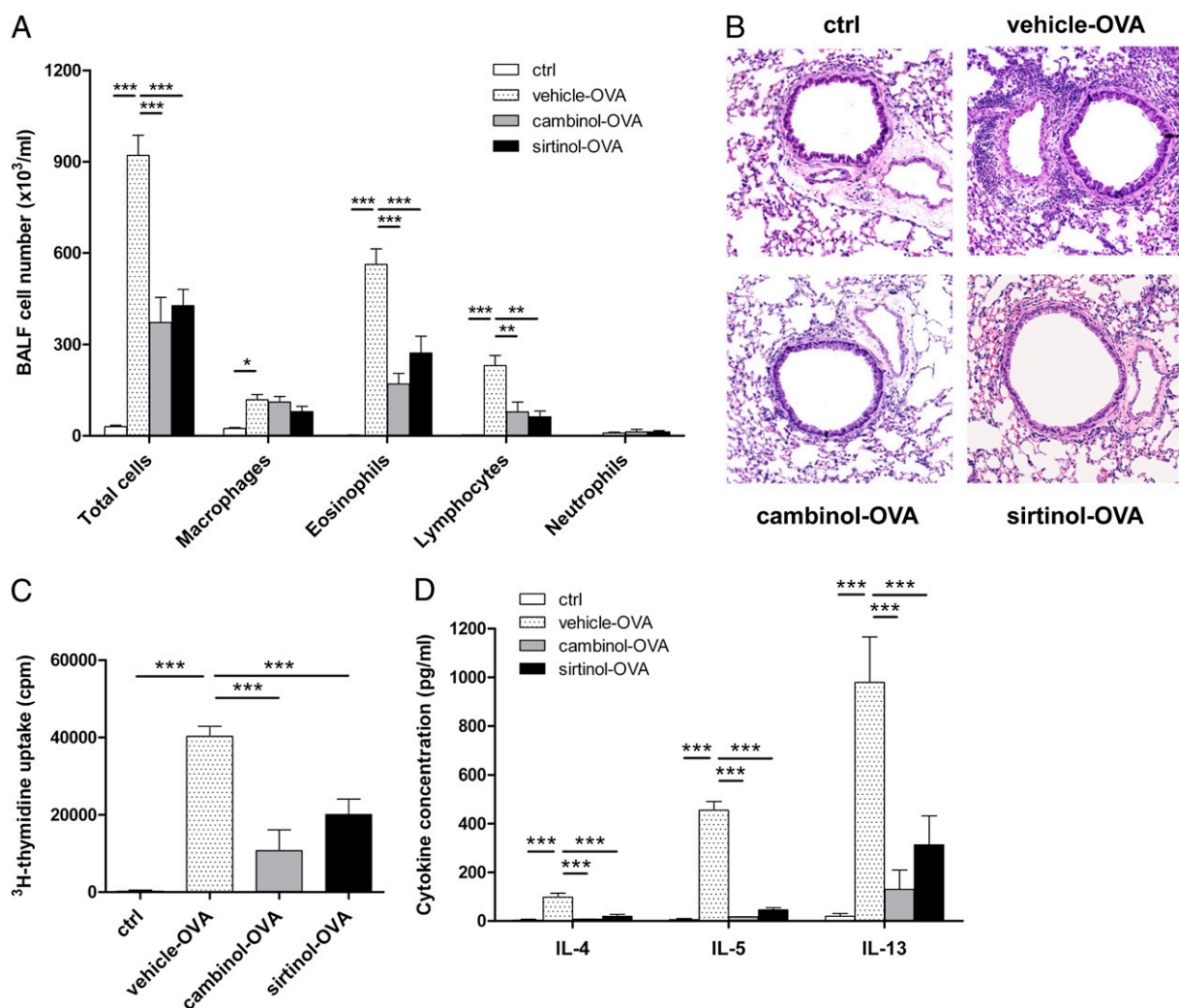


FIGURE 4. Cambinol- and sirtinol-treated DCs have decreased ability to induce Th2 responses in the lung. *A–D*, On day 0, naive BALB/c mice were injected i.t. with vehicle-treated unpulsed BMDCs (ctrl), vehicle-treated OVA-pulsed BMDCs (vehicle-OVA), cambinol-treated OVA-pulsed BMDCs (cambinol-OVA), or sirtinol-treated OVA-pulsed BMDCs (sirtinol-OVA). From days 10–14, mice were exposed to OVA aerosols. Twenty-four hours after the last challenge, the severity of airway allergy was evaluated. *A*, Total and differential cell counts in the BALF. *B*, H&E-stained lung sections (original magnification $\times 100$). *C*, T cell proliferation estimated by measuring [^3H]thymidine uptake during the last 16 h of a 3-d *in vitro* culture of BLN cells with OVA. *D*, ELISA for cytokines in the BLN cell-culture supernatants. Data shown are representative of four independent experiments using six mice in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

circulating DCs, T cells, and B cells, as well as the distribution of these cell types in nondraining axillary and inguinal LNs, were unaffected in cambinol- and sirtinol-treated mice compared with OVA/vehicle-OVA animals (data not shown).

To unambiguously demonstrate an effect of cambinol and sirtinol on the migratory capacity of lung DCs, we adapted an assay developed by Vermaelen et al. (38). In this assay, 800 μ g FITC-labeled OVA was given i.t. to vehicle-, cambinol-, or sirtinol-treated mice, and migrating lung DCs were counted in BLNs 24 h later by flow cytometry. As shown in Supplemental Fig. 2A and 2B, the number of Ag-loaded DCs was significantly lower in the BLNs of cambinol- and sirtinol-treated mice than in those of vehicle-treated controls, confirming an inhibitory effect of these drugs on lung DC migration.

Induction of robust Th2 immune responses in the airways requires not only the migration of allergen-loaded lung DCs to the BLNs, but also their maturation (39). We thus examined whether treatment with cambinol and sirtinol could alter the maturation process in lung DCs. For that, lung DCs were FACS sorted and stimulated ex vivo with OVA in the presence or absence of sirtuin inhibitors. Of note, the commercial batch of OVA we used in the current study contained enough endotoxin to induce full DC maturation. As expected, OVA-stimulated lung DCs, compared with freshly isolated lung DCs, displayed increased expression of the maturation markers CD40, CD80, and CD86 (Fig. 2D). Expression of these markers was substantially reduced when the cells were treated with cambinol or sirtinol before OVA stimulation (Fig. 2D). Consistent with our results on DC migration, expression of CCR7, a chemokine receptor playing a pivotal role in DC trafficking (40), was significantly decreased when OVA-stimulated lung DCs were treated with sirtuin inhibitors (Fig. 2D). To ascertain that these observations were not due to toxic effects of cambinol and sirtinol, apoptosis was measured in treated and untreated lung DCs. Fig. 2E shows that neither cambinol nor sirtinol induced significant apoptosis in these cells.

Our data on DC maturation implied that sirtuin inhibition was able to compromise the ability of lung DCs to stimulate T cells. To further address this point, we measured the effects of cambinol and sirtinol on lung DC-induced proliferation of OVA-specific T cells in vitro. For this purpose, we used CD4⁺ T cells from OVA-specific, TCR-transgenic DO11.10 mice (41). Fig. 3A shows that treatment of OVA-pulsed lung DCs with sirtuin inhibitors significantly reduced their ability to induce DO11.10 T cell proliferation. Moreover, DO11.10 T cells stimulated with cambinol- or sirtinol-treated, OVA-pulsed lung DCs secreted lower amounts of Th2 cytokines than those stimulated with vehicle-treated control DCs (Fig. 3B), clearly demonstrating that sirtuins are required for efficient activation of Th2 cells by lung DCs.

To definitively prove that sirtuin inhibition prevents DC-driven Th2 responses in the lung, we used a model in which i.t. transfer of OVA-pulsed BMDCs leads to allergic airway inflammation upon subsequent OVA aerosol challenge (39). BMDCs were used because the number of DCs obtained from the lungs of mice was too small to perform these large-scale experiments. This model is relevant in the study of the biology of DCs in airway allergy, because it was recently shown that BMDCs display a phenotype very similar to that of lung DCs upon allergenic challenge of the airways (42). Further supporting that this model is relevant to the current study, we demonstrated that the ability of BMDCs to mature and stimulate DO11.10 T cells in vitro was abolished upon cambinol and sirtinol treatment (Supplemental Fig. 3A–D), as shown for lung DCs (Fig. 3). Moreover, BMDCs expressed the same sirtuins as lung DCs, namely Sirt1, Sirt2, and Sirt7, along

with low levels of Sirt3, Sirt5, and Sirt6 (Supplemental Fig. 4). As previously shown by Lambrecht et al. (39), mice that were immunized with OVA-pulsed, vehicle-treated BMDCs developed BALF and peribronchial eosinophilia following OVA challenge, an effect not observed in mice that were injected with unpulsed, vehicle-treated BMDCs (Fig. 4A, 4B). The pretreatment of OVA-pulsed BMDCs with either cambinol or sirtinol significantly reduced their capacity to induce the development of allergic airway inflammation (Fig. 4A, 4B). These effects of cambinol and sirtinol were accompanied by a reduced capacity of BLN T cells to proliferate and produce Th2 cytokines following OVA restimulation (Fig. 4C, 4D).

Altogether, these results demonstrate that sirtuin inhibition impairs maturation and migration of lung DCs, thereby dampening DC-driven Th2 priming and allergic airway inflammation.

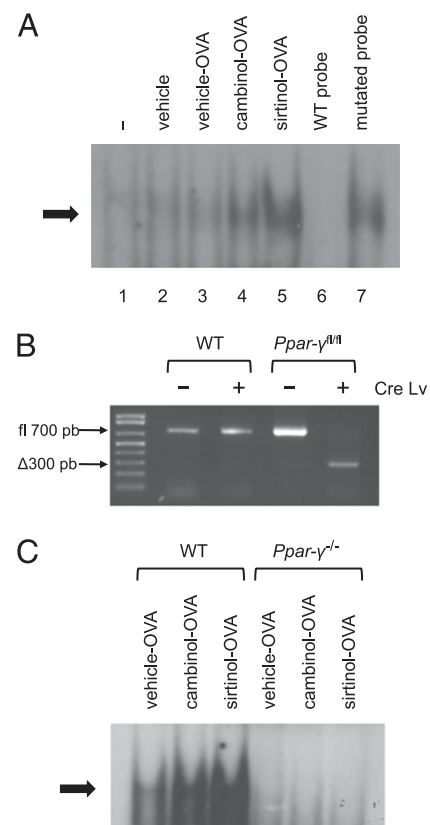


FIGURE 5. Activation of PPAR- γ by sirtuin inhibition in DCs and generation of PPAR- γ -deficient DCs. **A**, EMSA for PPAR- γ DNA-binding activity in BMDCs from naive BALB/c mice pulsed with OVA in the presence of cambinol (lane 4), sirtinol (lane 5), or vehicle alone (lane 3) for 6 h. We used unpulsed BMDCs treated with vehicle alone (lane 2) or left untreated (lane 1) as controls. The solid arrow indicates specific PPAR- γ complexes. We confirmed the specificity of PPAR- γ complexes by competition experiments in which we incubated the nuclear extract used in lane 4 with a 50-fold excess of unlabeled WT (lane 6) or mutated (lane 7) probes. **B**, PPAR- γ ^{fl/fl} BMDCs were generated by transducing PPAR- γ ^{fl/fl} BMDCs with a lentiviral vector encoding the Cre recombinase under the control of the PGK promoter (Cre Lv). We used WT BMDCs from C57BL/6 mice transduced with the same vector as controls. Cre-mediated recombination was determined by RT-PCR of floxed (700 bp) and deleted (300 bp) PPAR- γ transcripts. **C**, PPAR- γ DNA-binding activity in WT and PPAR- γ ^{fl/fl} BMDCs pulsed with OVA in the presence of cambinol, sirtinol, or vehicle alone for 6 h. The solid arrow indicates specific PPAR- γ complexes. Data shown are representative of four independent experiments.

Cambinol and sirtinol exert their anti-inflammatory effects in DCs through the derepression of PPAR- γ

Sirtuins have been shown to be capable of repressing PPAR- γ (43, 44), a nuclear receptor mainly involved in the regulation of carbohydrate and lipid metabolism (45). Given that activation of PPAR- γ in DCs renders them unable to prime Th2 responses in the airways (46), we hypothesized that abrogation of sirtuin-mediated PPAR- γ repression could be the molecular mechanism by which sirtuin inhibitors exert their anti-inflammatory effects in lung DCs.

To test our hypothesis, we first assessed the activity of PPAR- γ by EMSA in vehicle-, cambinol-, and sirtinol-treated, OVA-pulsed DCs. As shown in Fig. 5A (lanes 1–5), treatment of OVA-pulsed BMDCs with sirtuin inhibitors led to a strong increase in PPAR- γ DNA-binding activity, whereas treatment of unpulsed or OVA-pulsed DCs with vehicle alone had no effect on basal PPAR- γ activity. DNA-binding competition experiments using 50-fold excess of unlabeled WT and mutated probes confirmed the specificity of PPAR- γ binding (Fig. 5A, lanes 6 and 7).

To demonstrate that PPAR- γ activation was responsible for the anti-inflammatory effects of sirtuin inhibitors, it was necessary to show that cambinol and sirtinol were no longer able to inhibit DC function in the absence of PPAR- γ . We thus generated PPAR- γ -deficient DCs by transducing *Ppar- γ* -floxed (*Ppar- γ ^{fl/fl}*) BMDCs with an advanced generation lentiviral vector carrying the Cre recombinase cDNA under the control of the PGK promoter. WT BMDCs transduced with the same lentiviral vector were used as controls. Efficient Cre-mediated recombination in *Ppar- γ ^{fl/fl}* cells results in deletion of exons 1 and 2 of the *Ppar- γ* gene and therefore in shortening of the PPAR- γ transcript [300 bp for the recombinant form versus 700 bp for the floxed form (47)]. The short transcript was predominant in Cre recombinase-transduced *Ppar- γ ^{fl/fl}* BMDCs, whereas only the full-length transcript was detected in control cells, confirming that this lentiviral transduction method allowed the generation of *Ppar- γ ^{-/-}* BMDCs (Fig. 5B). Moreover, the DNA binding activity of PPAR- γ did not increase in Cre recombinase-transduced *Ppar- γ ^{fl/fl}* BMDCs following treatment with cambinol or sirtinol, demonstrating a loss of PPAR- γ function in these cells (Fig. 5C). It has to be noted that no shifted bands were detected when nuclear extracts from *Ppar- γ ^{-/-}* BMDCs, treated or not with cambinol or sirtinol, were used (Fig. 5C), confirming the specificity of the EMSA complexes.

The functional experiments described above for WT DCs were repeated with *Ppar- γ ^{-/-}* DCs to prove the involvement of this NF in the anti-inflammatory effects of sirtuin inhibitors. As shown in Fig. 6, cambinol and sirtinol failed to diminish the capacity of

OVA-pulsed *Ppar- γ ^{-/-}* BMDCs to stimulate OVA-specific T cells in vitro, whereas they did in control experiments. Moreover, pretreatment of OVA-pulsed *Ppar- γ ^{-/-}* BMDCs with either cambinol or sirtinol did not reduce their ability to induce allergic airway inflammation following i.t. transfer (Fig. 7).

These data show that sirtuin inhibitors impair DC function and therefore the development of asthmatic responses by derepressing the anti-inflammatory factor PPAR- γ .

Sirt1 is required for the repression of PPAR- γ and optimal pro-Th2 accessory activity of DCs

We finally sought to determine which of the sirtuins is required for the pro-Th2 activity of DCs in allergic conditions. Sirtinol has first been reported to selectively inhibit Sirt2 NAD-dependent deacetylase activity in vitro with an IC₅₀ value of 58 μ M (26). However, further studies demonstrated that sirtinol also antagonizes Sirt1, although the IC₅₀ value in this case was higher (131 μ M) (27). Cambinol inhibits Sirt1 and Sirt2 with similar IC₅₀ values of 56 and 59 μ M, respectively (29). Therefore, although cambinol has been reported to have a very weak inhibitory activity against Sirt5 (42% inhibition at 300 μ M) (29), the activity of sirtinol and cambinol seems to be principally restricted to Sirt1 and Sirt2. This, coupled with the fact that Sirt1 is the main sirtuin known to repress PPAR- γ (43), made Sirt1 the most likely candidate implicated in DC-mediated induction of Th2 responses in the airways.

We therefore investigated the possible involvement of Sirt1 in the pro-Th2 activity of DCs. *Sirt1*^{-/-} mice display an elevated postnatal lethality attributable to developmental problems (48, 49), which precludes their use in models of induced allergy. We therefore generated mice conditionally deficient for Sirt1 in DCs (*Sirt1* cKO) by crossing mice with a floxed *Sirt1* gene (*Sirt1*^{fl/fl} mice) (50) with transgenic mice expressing the Cre recombinase under the dependence of the *Cd11c* promoter (CD11c-Cre mice) (51). *Sirt1*^{fl/fl} and CD11c-Cre mice were used as control. Because both controls gave comparable results, only those in *Sirt1*^{fl/fl} are shown. In the model of induced airway allergy, *Sirt1* cKO mice displayed significantly reduced parameters of airway inflammation compared with control mice (Fig. 8). This correlated with decreased migratory activity of lung DCs upon Ag exposure of the airways (Fig. 9A). Finally, when assessing whether Sirt1 intervenes in the repression of PPAR- γ in DCs under pro-Th2 conditions, EMSAs revealed that PPAR- γ activity was significantly upregulated in *Sirt1* cKO DCs compared with control DCs (Fig. 9B).

These results thus identify Sirt1 as a sirtuin implicated in the repression of PPAR- γ in lung DCs and the subsequent promotion of a pro-Th2 phenotype of these cells in allergic conditions.

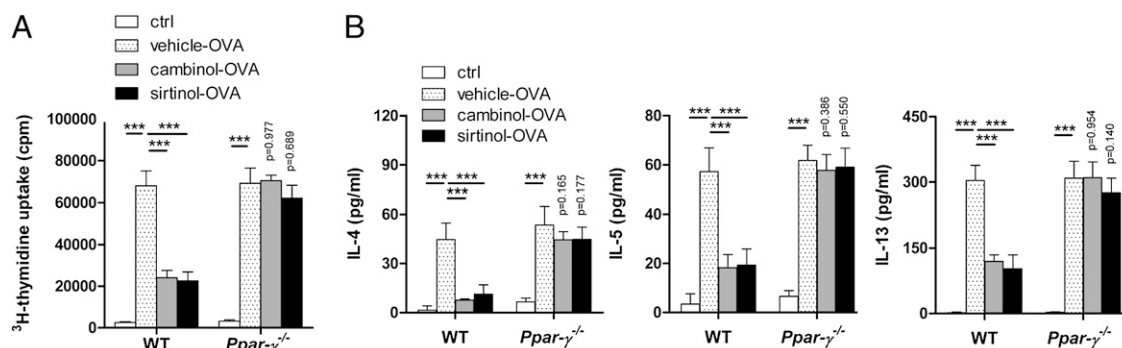


FIGURE 6. Cambinol and sirtinol are unable to affect the stimulation of Ag-specific T cells by *Ppar- γ ^{-/-}* DCs. A and B, WT and *Ppar- γ ^{-/-}* BMDCs from naive C57BL/6 mice were stimulated for 16 h with OVA in the presence of vehicle, cambinol, or sirtinol. BMDCs were then extensively washed, irradiated, and cocultured for 3 d with OVA-specific OT-II CD4⁺ T cells. We used unpulsed, vehicle-treated BMDCs as controls (ctrl). A, T cell proliferation evaluated by measuring [³H]thymidine uptake during the last 16 h of culture. B, ELISA for cytokines in the culture supernatants. Data shown are representative of three independent experiments. ****p* < 0.001.

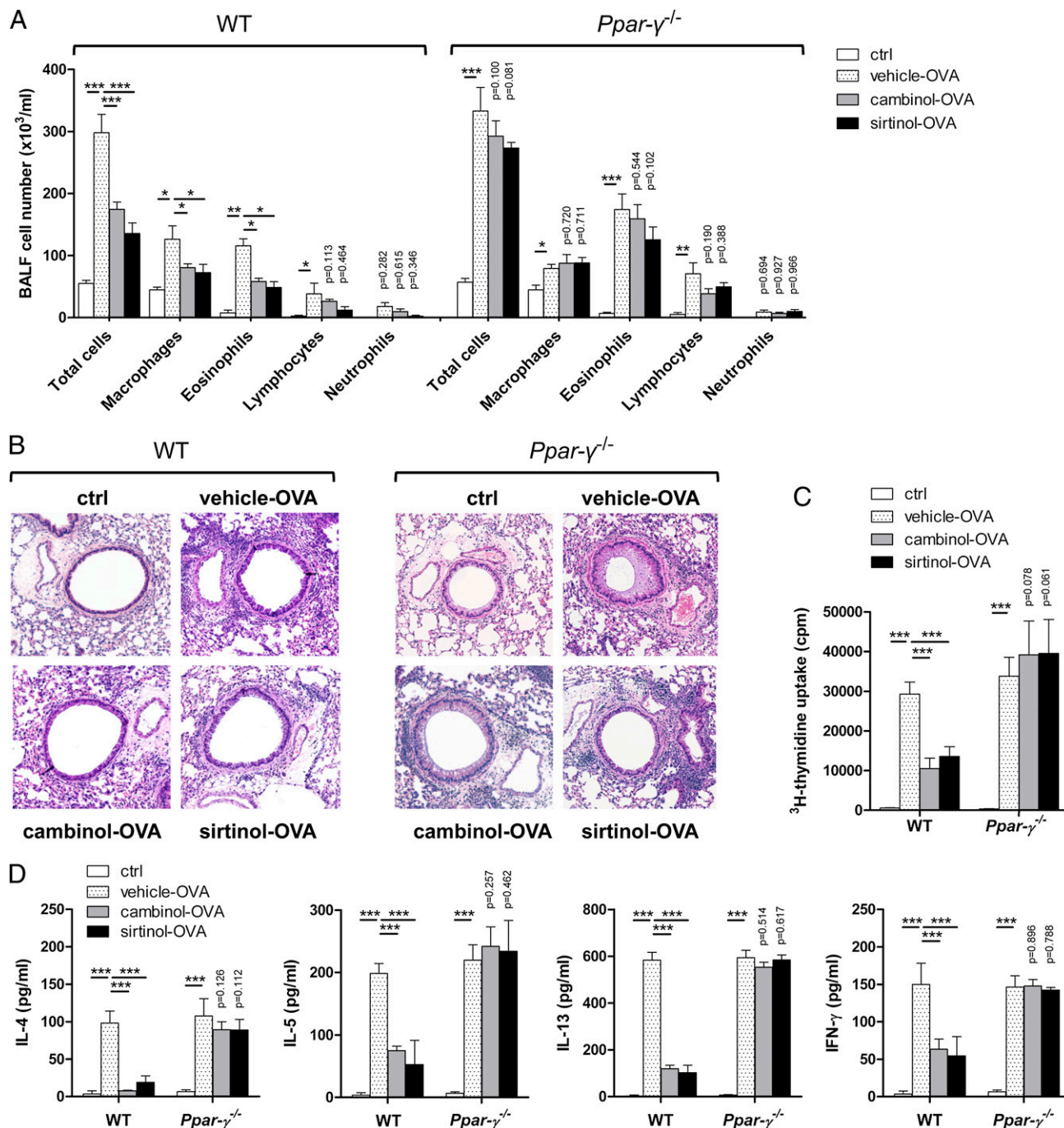


FIGURE 7. Cambinol- and sirtinol-treated *Ppar-γ^{-/-}* DCs retain the capacity to induce airway allergy. **A–D.** On day 0, naive C57BL/6 mice were injected i.t. with vehicle-treated unpulsed BMDCs (ctrl), vehicle-treated OVA-pulsed BMDCs, cambinol-treated OVA-pulsed BMDCs, or sirtinol-treated OVA-pulsed WT or *Ppar-γ^{-/-}* BMDCs. From days 10–14, mice were exposed to OVA aerosols. We evaluated the severity of airway allergy 24 h after the last challenge. **A**, Total and differential cell counts in the BALF. **B**, H&E-stained lung sections (original magnification $\times 100$). **C**, T cell proliferation estimated by measuring [^3H]thymidine uptake during the last 16 h of a 3-d in vitro culture of BLN cells with OVA. **D**, ELISA for cytokines in the BLN cell-culture supernatants. Data shown are representative of three independent experiments using six to eight mice in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Discussion

The sirtuins are a highly conserved family of NAD⁺-dependent enzymes that are known to regulate diverse biological functions, including aging, cellular stress resistance, tumorigenesis, and energy metabolism (7–9). In vitro studies have demonstrated that sirtuins may also dampen the inflammatory response by inhibiting NF- κ B and AP-1 (14–18), two proinflammatory transcription factors. Given that NF- κ B and AP-1 are both critically involved in the effector phase of allergic airway inflammation (52, 53), it may

have been expected that the asthmatic reaction would be aggravated by the inhibition of sirtuins. Yet, our data, in agreement with a recent report (28), indicate that treatment of mice with sirtuin inhibitors decreases the parameters of allergic airway inflammation in a mouse model of allergic asthma.

In their report, Kim et al. (28) suggested that the anti-inflammatory effects of sirtinol on airway allergy were due to the impairment of the activation by Sirt1 of hypoxia-inducible factor (HIF)-1 α , a transcription factor activated during, and regulator of,

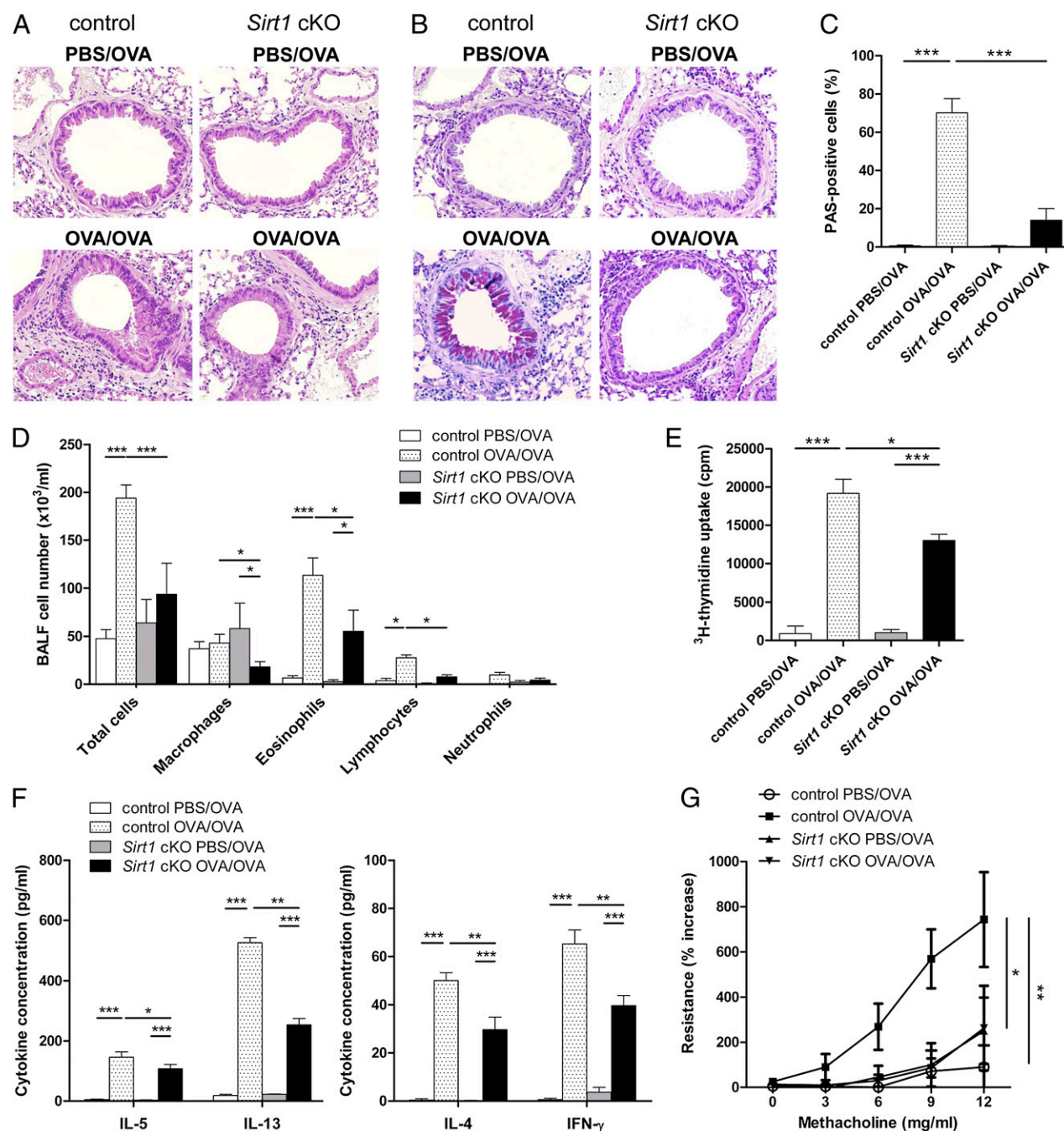


FIGURE 8. *Sirt1* cKO mice are partially protected from airway allergy. **A**, H&E-stained lung sections of control and *Sirt1* cKO mice sensitized or not to OVA and exposed to aerosolized OVA (original magnification $\times 100$). **B**, Representative PAS-stained lung section (original magnification $\times 100$). **C**, Quantification of mucus production in control and *Sirt1* cKO mice sensitized or not to OVA and exposed to aerosolized OVA (original magnification $\times 100$). **D**, Total and differential cell counts in the BALF recovered from the animals. **E**, T cell proliferation estimated by measuring [^{3} H]thymidine uptake during the last 16 h of a 3-d in vitro culture of BLN cells with OVA. **F**, ELISA for cytokines in the BLN cell-culture supernatants. **G**, AHR to increasing doses of methacholine assessed by invasive measurement of dynamic resistance. Data shown are representative of three independent experiments using six to eight mice in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

inflammatory responses (54). Yet, the authors only used a pharmacological approach and a single sirtuin inhibitor, sirtinol, which is known to have higher inhibitory potential on Sirt2 than on Sirt1 (27). The sirtuin implicated in the observed anti-inflammatory effects of sirtinol thus was not formally identified. Furthermore, the evidence that sirtinol downregulates HIF-1 α activity in this model was purely correlative, and it cannot be ruled out that the observed reduction in HIF-1 α activity was a collateral effect of the downregulation of inflammation by other mechanisms.

Consistent with the existence of such HIF-1 α -independent mechanisms, our data indeed indicate that sirtuin inhibition impairs the optimal reactivation of Th2 responses upon allergen challenge of the airways through PPAR- γ -dependent mechanisms. Because Th2 responses are the essential drivers of allergic airway inflammation, it is thus most likely that inhibition of these adaptive responses is a major mechanism by which cambinol and sirtinol suppress all of the characteristic features of asthmatic inflammation.

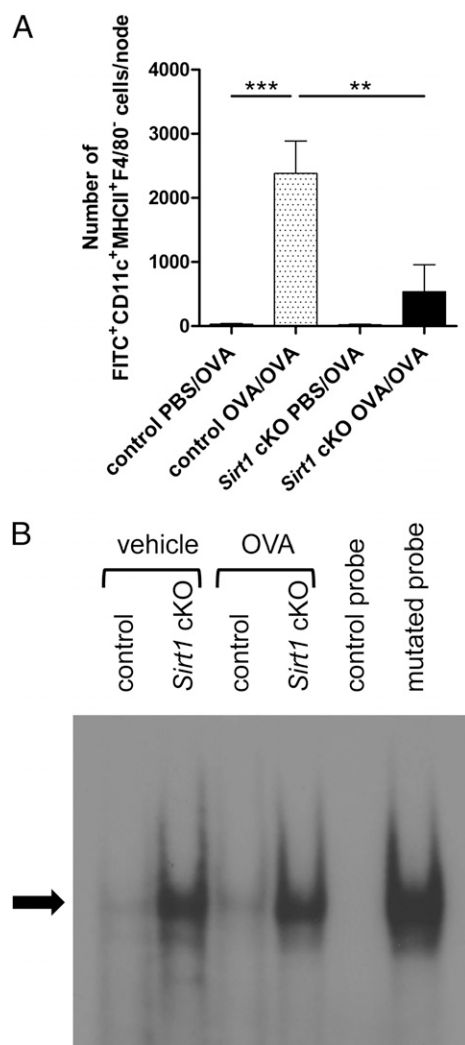


FIGURE 9. Sirt1 deficiency in lung DCs impairs their migration and derepresses PPAR- γ activity. **A**, Flow cytometric assessment of the number of OVA-loaded DCs (FITC⁺CD11c⁺MHC II⁺F4/80⁻ cells) in the BLNs of control and *Sirt1* cKO mice 24 h after i.t. administration of FITC-labeled OVA. **B**, PPAR- γ DNA-binding activity in BMDCs from control and *Sirt1* cKO mice pulsed with OVA or PBS vehicle for 6 h. The solid arrow indicates specific PPAR- γ complexes. We confirmed the specificity of PPAR- γ complexes by competition experiments in which we incubated the nuclear extract used in lane 4 with a 50-fold excess of unlabeled WT (lane 5) or mutated (lane 6) probes. Data shown are representative of three independent experiments. ** p < 0.01, *** p < 0.001.

We also formally identify Sirt1 as a sirtuin promoting Th2 responses through its inhibitory effect on PPAR- γ activity in DCs. Yet, the inhibition of airway inflammation observed in *Sirt1* cKO mice was slightly lower than the one observed with pharmacological sirtuin inhibition (compare Fig. 1A–C with 8A–D). Our data thus support that Sirt1 activity in DCs promotes adaptive Th2 responses in allergic conditions and that this pathway is an important contributor to allergic airway inflammation, but do not rule out that Sirt1 and/or Sirt2 may have additional proinflammatory effects in other immune cells implicated in this type of inflammation. For instance, we observed that sirtinol and cambinol inhibit proliferation and cytokine secretion of Th2 cells polarized and activated in vitro, without affecting cell survival (A. Mayer and F. Andris, unpublished observations). Sirtuins might thus also regulate T cell responses directly, although this hypothesis will require further testing.

As illustrated by the above considerations, the role of sirtuins in immune cells remains largely unknown. In particular, whether sirtuins are expressed in DCs and whether they modulate DC function had never been investigated. In the present report, we showed that lung DCs, which are necessary and sufficient for Th2 cell stimulation during ongoing allergic airway inflammation (37, 55), express mainly Sirt1, Sirt2, and Sirt7. Moreover, we demonstrated that pharmacological inhibition of sirtuins impairs allergen-induced maturation and migration of lung DCs, thereby downregulating Th2 cell activation. Using conditional genetic deletion, we furthermore showed that the effects of sirtuin inhibitors on adaptive Th2 responses depended on Sirt1 activity in DCs. Finally, we could demonstrate that Sirt1 inhibits PPAR- γ activity in DCs. PPAR- γ is a key regulator of carbohydrate and lipid metabolism (45), but growing evidence indicates that PPAR- γ also plays an important role in controlling immune and inflammatory responses, including allergic airway inflammation. Indeed, the PPAR- γ agonists ciglitazone, rosiglitazone, and GI 262570 display beneficial effects in various animal models of allergic asthma at least in part due to the capacity of PPAR- γ to reduce maturation, migration, and immunogenicity of DCs (56–60). Thus, given that most of the effects of sirtuin inhibitors were demonstrated on highly purified DCs and were similar to the effects observed in mice conditionally deficient for Sirt1 in their DCs, it is evident that Sirt1 acts in a cell-autonomous manner to control DC function. Our study mainly focused on the role of sirtuins in the induction of Th2 responses by lung DCs. We nevertheless also observed that treatment of keyhole limpet hemocyanin-loaded BMDCs with cambinol or sirtinol prior to footpad inoculation in naive recipient mice inhibits the induction by these cells of both Th1 and Th2 responses in the draining lymph nodes (T. Marichal, unpublished observations). Sirtuin activity might thus be generally required for the activity of DCs as APCs. Therefore, it will be worthwhile studying whether sirtuins are also expressed in other DC populations and whether and how these enzymes participate in the induction of Th1 and Th17 responses by DCs in vivo.

An intriguing question is how sirtuins exert both pro- and anti-inflammatory functions. As mentioned above, Sirt1 seems to play an anti-inflammatory role in COPD (15, 17). COPD is a chronic inflammatory disease of the lungs mainly caused by repeated exposure to cigarette smoke, and there is accumulating evidence that accelerated aging of lungs in response to cigarette smoke-induced oxidative stress is critically implicated in the pathogenesis and progression of this disease (6, 61). It has been demonstrated that sirtuins confer cellular resistance to oxidative stress (9, 12). This increased stress resistance comes from the interaction between sirtuins and members of the forkhead box class O (FOXO) family of transcription factors. Sirt1 can bind and deacetylate FOXO3a, leading to a selective upregulation of FOXO-regulated stress resistance genes (62, 63). In addition, Sirt1 can also protect cells against oxidative stress by regulating the heat shock response and inhibiting p53 transcriptional activity (64–66). It is therefore probable that sirtuins prevent the development of COPD not only by antagonizing NF- κ B but also by attenuating the deleterious effects of cigarette smoke-induced oxidative stress. Sirtuins have also been reported to play an anti-inflammatory role in metabolic diseases. For example, transgenic *Sirt1* mice under a high-fat diet display lower lipid-induced inflammation than WT controls (19). Moreover, sirtuins improve insulin sensitivity in adipocytes through their anti-inflammatory properties (18). It thus appears that sirtuins are protective in inflammatory diseases related to aging, oxidative stress, and metabolic dysfunction. Unlike allergic airway inflammation, these inflammatory conditions do not essentially depend on Ag-specific

T cell responses and rather are mainly driven by innate inflammatory mechanisms. As we have demonstrated that sirtuins are required for optimal adaptive Th2 responses, one may speculate that sirtuins could promote T cell-dependent inflammations, such as airway allergy, but rather dampen more T cell-independent inflammatory conditions, such as COPD.

The underlying pathogenic mechanisms in asthma and airway allergy are only partly understood, a situation reflected in the variable and incomplete responses by patients to current therapeutics. The emerging concept that targeting airway DC function might be a powerful method to efficiently treat airway allergy has recently been validated by the group of Lambrecht (67, 68). The present study demonstrates for the first time, to our knowledge, that sirtuins (i.e., Sirt1) promote allergic airway inflammation by boosting lung DC function. We therefore suggest that blocking sirtuin activity in lung DCs may have therapeutic potential in the treatment of airway allergy.

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Disclosures

The authors have no financial conflicts of interest.

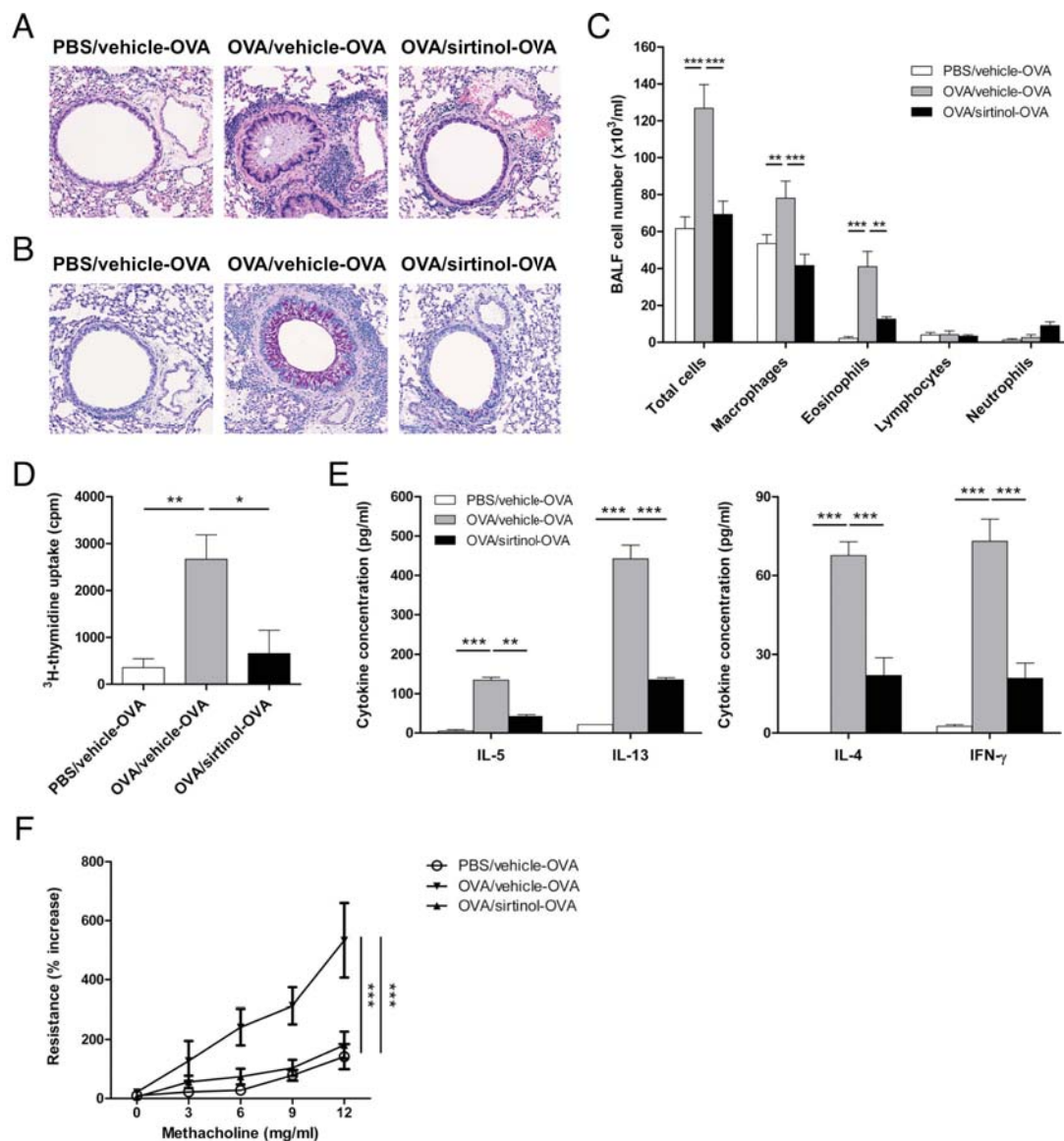
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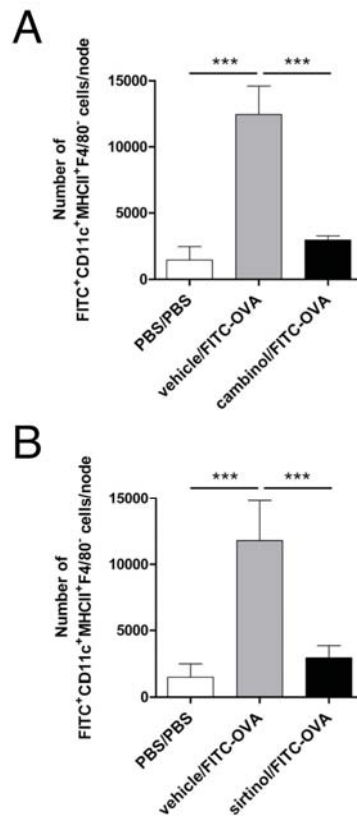
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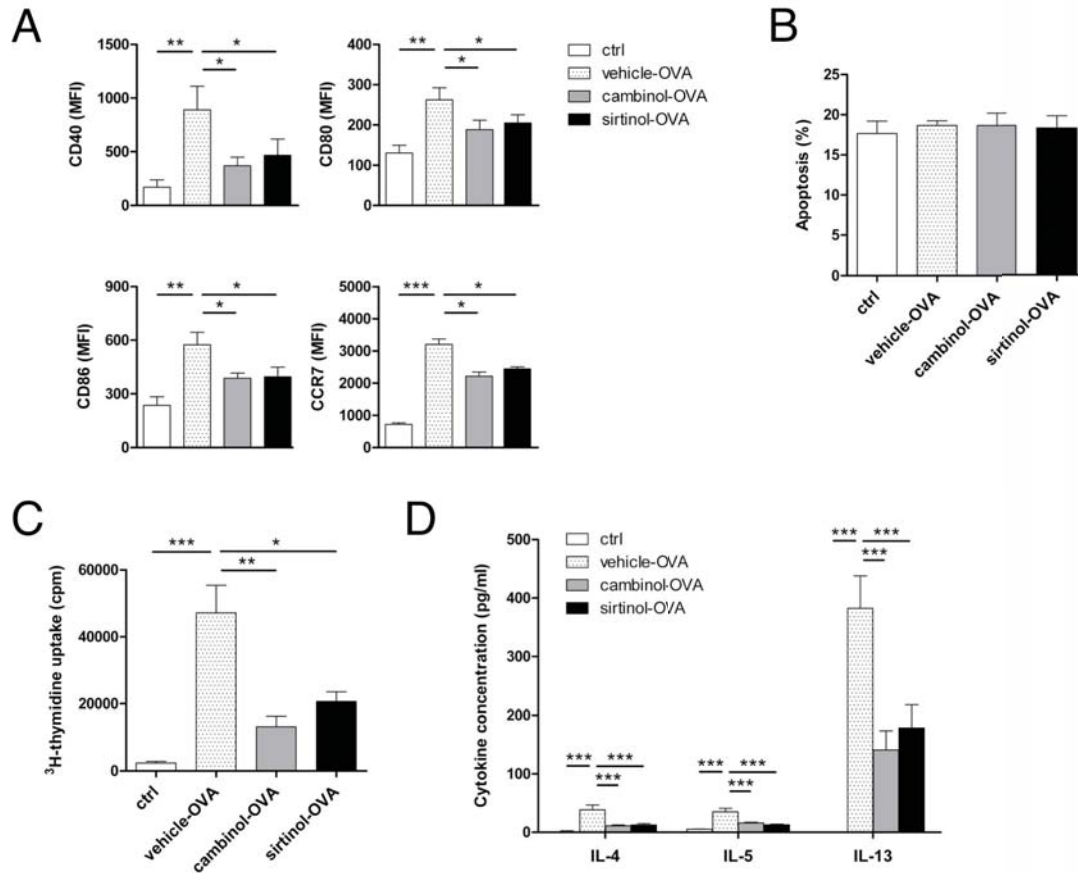
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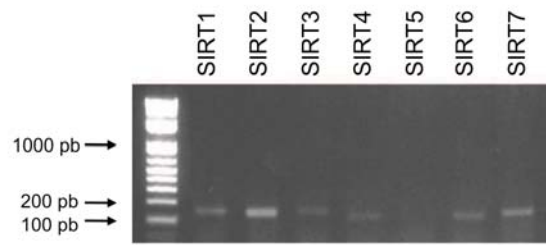
Supplemental Figure 1. Treatment with sirtinol markedly attenuates the features of airway allergy. A and B, Hematoxylin and eosin (A) or periodic acid-Schiff (B)-stained lung sections of PBS/vehicle-OVA, OVA/vehicle-OVA and OVA/sirtinol-OVA BALB/c mice (original magnification X100). C, Total and differential cell counts in the bronchoalveolar lavage fluid (BALF). D, T cell proliferation estimated by measuring ³H-thymidine uptake during the last 16 h of a 3-day *in vitro* culture of bronchial lymph node (BLN) cells with OVA. E, ELISA for cytokines in the BLN cell culture supernatants. F, Airway hyperresponsiveness to increasing doses of methacholine assessed by invasive measurement of dynamic resistance. Data shown are representative of three independent experiments using 6-8 mice in each group. *, P < 0.05; **, P < 0.01; ***, P < 0.001. (cpm: counts per minute).



Supplemental Figure 2. Cambinol and sirtinol inhibit lung DC migration. A and B, FITC-labeled OVA was instilled into the trachea of BALB/c mice that had been treated i.p. with cambinol (A) or sirtinol (B) 18 and 3 h before. 24 h later, bronchial lymph nodes were analyzed by flow cytometry for the presence of OVA-loaded DCs (FITC⁺CD11c⁺MHCII⁺F4/80⁺ cells). Control mice were treated with either PBS or vehicle alone. Data shown are representative of three independent experiments using 6-8 mice in each group. ***, P < 0.001.



Supplemental Figure 3. Sirtuin inhibition impairs the maturation and stimulatory activity toward T cells of bone marrow-derived DCs. A and B, 10^6 BMDCs were stimulated for 10 h with OVA in the presence of either vehicle, cambinol, or sirtinol. A, Flow cytometric assessment of the expression of CD40, CD80, CD86, and CCR7 on the BMDCs. Untreated BMDCs were used as controls (ctrl). Mean fluorescence intensities (MFIs) are provided. B, Percentage of apoptotic BMDCs assayed using dual-color annexin-V-FITC/propidium iodide staining and flow cytometry analyses. C and D, BMDCs were stimulated for 16 h with OVA in the presence of either vehicle, cambinol, or sirtinol. BMDCs were then extensively washed, irradiated, and cocultured for 3 days with OVA-specific DO11.10 CD4⁺ T cells. Unpulsed, vehicle-treated BMDCs were used as controls (ctrl). C, T cell proliferation evaluated by measuring ³H-thymidine uptake during the last 16 h of culture. D, ELISA for cytokines in the culture supernatants. Data shown are representative of three independent experiments ($n = 6$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.



Supplemental Figure 4. Sirtuin expression in BMDCs. RT-PCR analysis of the expression of sirtuins (Sirt1-7) in BMDCs. Data shown are representative of three independent experiments.