

Letter to the Editor

Overexpression of sirtuin 6 suppresses allergic airway inflammation through deacetylation of GATA3

To the Editor:

Acetylation is one of the most well-studied posttranslational modifications of proteins, and a reciprocal relationship between histone acetyltransferase and histone deacetylase (HDAC) has been demonstrated in patients with asthma.¹ Furthermore, there is evidence that the anti-inflammatory activities of glucocorticoids are mediated by increased HDAC activity, suggesting that targeting HDAC activity could be a promising therapeutic intervention for asthma.² In the present study, we investigated the effect of increased HDAC activity mediated by overexpression of sirtuin 6 (Sirt6) on allergic airway inflammation (AAI) in mice.

To assess the role of sirtuins in AAI, protein levels of all sirtuins (Sirt1-7) in ovalbumin (OVA)-immunized and OVA-challenged mice were determined (see Fig E1 in this article's Online Repository at www.jacionline.org). Levels of Sirt1 and Sirt6 increased after the last OVA challenge (see Fig E2 in this article's Online Repository at www.jacionline.org). Because overexpression of Sirt1 only partially ameliorated AAI in mice (see Fig E3 in this article's Online Repository at www.jacionline.org), we focused on Sirt6 in the present study. Adenoviruses expressing Sirt6 (Ad-Sirt6), catalytically inactive Sirt6-H133Y (AdmSirt6), or saline were instilled intratracheally 3 days before the first OVA

challenge (see Fig E4 in this article's Online Repository at www.jacionline.org). Overexpression of Sirt6 significantly attenuated the infiltration of OVA challenge-induced inflammatory cells, such as eosinophils, lymphocytes, and macrophages, into lung tissues (Fig 1, A and B; see Fig E5 in this article's Online Repository at www.jacionline.org). Bronchiolar mucin accumulation, most commonly associated with a chronic asthmatic response,³ was effectively reduced to near-normal level by Ad-Sirt6 instillation, whereas excessive mucin production was obvious in the airways of AdmSirt6- as well as saline- or AdLacZ-instilled mice (Fig 1, C; Fig E5). The mRNA level of Muc5ac correlated well with the histological data (Fig E5). The level of eotaxin, which is a specific chemoattractant for eosinophils, was significantly lower in the bronchoalveolar lavage fluid and lung tissues of AdSirt6 mice compared with AdLacZ and AdmSirt6 mice (Fig 1, D). Transcript levels of CCL2, CCL3, and RANTES in lung tissues were also significantly downregulated by Sirt6 overexpression (see Fig E6 in this article's Online Repository at www.jacionline.org). Consistent with this, serum levels of IgE and IgG₁ were markedly lower in AdSirt6-instilled mice than in AdLacZ-instilled mice (Fig 1, E). In addition, Sirt6 overexpression protected OVA-challenged mice from airway hyperresponsiveness to methacholine, an airway dysfunction caused by AAI (Fig 1, F). Many features of experimentally induced AAI as well as asthma are thought to reflect the consequences of T_H2-cell-mediated immune responses through secretion of IL-4, IL-5, and IL-13.⁴ AdSirt6 instillation significantly

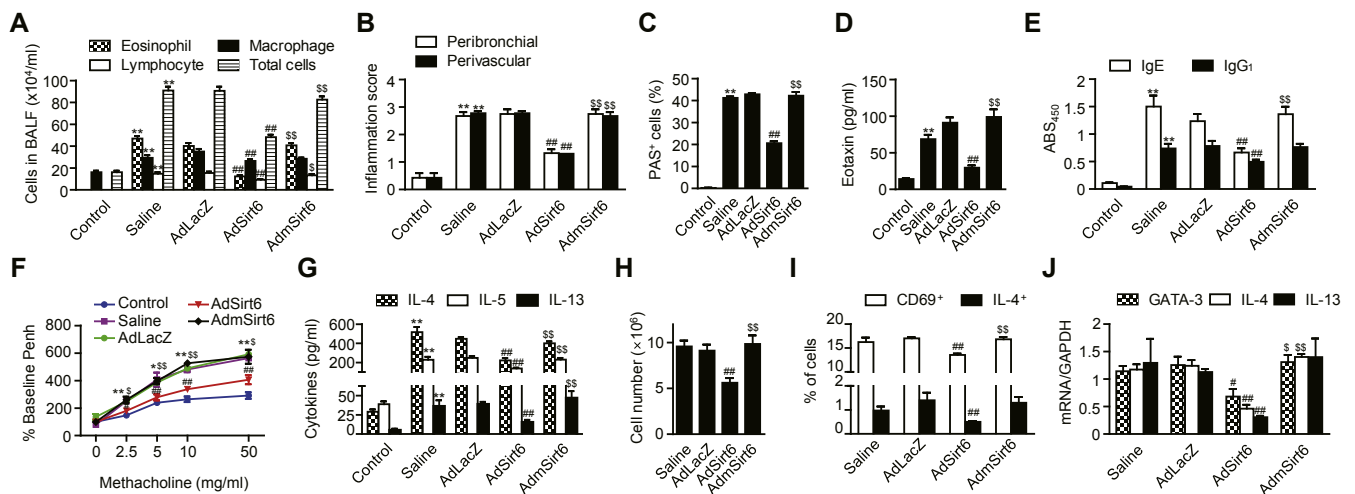


FIG 1. Overexpression of Sirt6 reduces AAI induced by OVA sensitization and challenge. **A**, Absolute cell numbers in BALF were counted ($n = 13$). **B**, Paraffin-embedded lung sections were stained with H&E, and perivascular and peribronchial inflammation were scored as described in this article's [Methods](http://www.jacionline.org) section in the Online Repository at www.jacionline.org ($n = 12$). **C**, Periodic acid-Schiff (PAS) staining was performed and PAS-positive cells were enumerated under light microscope ($n = 12$). **D**, Eotaxin levels in BALF were evaluated using ELISA ($n = 9$). **E**, OVA-specific IgE and IgG₁ levels in serum were measured using ELISA ($n = 10$). **F**, Airway hyperresponsiveness was assessed at 12 hours after the last OVA challenge. All animals were nebulized with various concentrations of methacholine as a bronchoconstrictor. Data are shown as percent increase in Penh relative to baseline ($n = 5$). **G**, Cytokine levels in BALF were measured using ELISA ($n = 10$). **H**, Cell numbers in dLNs are shown ($n = 8$). **I**, Single cells prepared from dLNs were analyzed for CD4⁺CD69⁺ cells or IL-4-producing CD4⁺ cells by flow cytometry ($n = 7$). **J**, dLNs were homogenized, and mRNA levels of GATA3 and T_H2 cytokines were analyzed by real-time RT-PCR ($n = 7$). Values are means $\pm$ SEMs. * $P < .05$ and ** $P < .01$ versus control; # $P < .05$ and ## $P < .01$ versus AdLacZ; \$ $P < .05$ and \$\$ $P < .01$ versus AdSirt6. BALF, Bronchoalveolar lavage fluid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

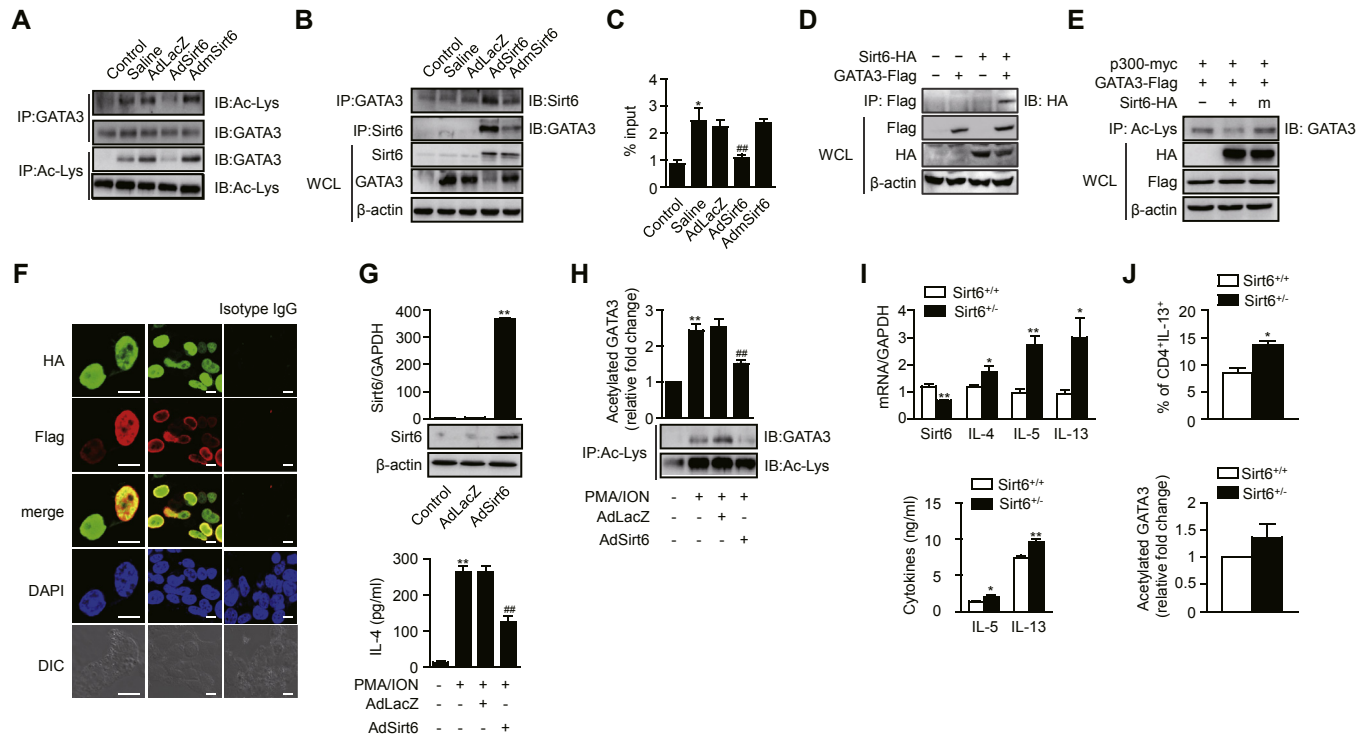


FIG 2. Sirt6 interacts with and deacetylates GATA3, and suppresses T_H2 immune responses. **A**, One hour after the last OVA challenge, proteins were extracted from lung homogenates. Acetylated GATA3 was evaluated through immunoprecipitation with anti-GATA3 antibody followed by immunoblotting with antiacetylated lysine antibody or *vice versa*. **B**, Lung lysates were immunoprecipitated with anti-GATA3 antibodies and immunoblotted with anti-Sirt6 antibody. **C**, IL-4 promoter occupancy by GATA3 was evaluated by the ChIP assay. DNA-GATA3 complexes were immunoprecipitated with anti-GATA3 antibody. Real-time RT-PCR was used to quantify the amount of precipitated IL-4 promoter DNA. Control IgG ChIP values were subtracted, and results were normalized to input DNA and are shown as the percentage of input DNA ($n = 4$). **D**, HEK293T cells were transfected with Sirt6-HA and/or GATA3-Flag plasmid, and the interaction between Sirt6 and GATA3 was analyzed using immunoprecipitation with anti-Flag antibody and immunoblot with anti-HA antibody. **E**, HEK293T cells were transfected with p300, GATA3-Flag, or Sirt6-HA plasmid, and acetylation of GATA3 was analyzed by immunoprecipitation. **F**, HEK293T cells transfected with Sirt6-HA and GATA3-Flag plasmids were fixed and stained with fluorescence antibodies, and the localization of Sirt6 and GATA3 was assessed by confocal microscopy. **G**, EL4 cell line was transduced with AdSirt6 or AdLacZ and then stimulated with PMA/ION for 48 hours. Sirt6 level was assessed using real-time RT-PCR and western blot (top) ($n = 6$). IL-4 concentration in the cell culture supernatants was measured by ELISA (bottom). **H**, Acetylation of GATA3 in EL4 nuclear protein extract was evaluated by ELISA and Co-IP ($n = 6$). **I**, CD4⁺ T cells were isolated from Sirt6^{+/+} or Sirt6^{-/-} mice and cultured under T_H2 polarization condition. mRNA levels of Sirt6, IL-4, IL-5, and IL-13 were analyzed by real-time RT-PCR (top), and IL-5 and IL-13 concentration in the cell culture supernatants were measured by ELISA (bottom) ($n = 4$). **J**, The proportion of CD4⁺ T cells producing IL-13 was evaluated with intracellular cytokine staining followed by flow cytometry analysis (top) ($n = 5$). Acetylation of GATA3 in CD4⁺ T-cell nuclear protein extract was evaluated by ELISA (bottom) ($n = 5$). ChIP, Chromatin immunoprecipitation; DAPI, 4'-6-diamidino-2-phenylindole, dihydrochloride; DIC, differential interference contrast; HA, hemagglutinin; ION, ionomycin; PMA, phorbol 12-myristate 13-acetate. Values are means $\pm$ SEMs. * $P < .05$ and ** $P < .01$ versus control; ## $P < .01$ versus AdLacZ.

decreased the levels of these cytokines in bronchoalveolar lavage fluid and lung tissues, indicating that Sirt6 overexpression attenuated T_H2 responses (Fig 1, G; see Fig E7 in this article's Online Repository at www.jacionline.org). Therefore, we further investigated whether Sirt6 affected the development of T_H2 responses in draining lymph nodes (dLNs) of the lung. dLNs of AdSirt6-instilled mice were substantially smaller in size and comprised significantly lower proportions of activated CD4⁺ T cells and IL-4-producing T_H2 cells compared with dLNs of control mice (Fig 1, H and I; see Fig E8 and Table E1 in this article's Online Repository at www.jacionline.org). Consistent with lung tissue analysis, AdSirt6 instillation caused a significant decrease in

mRNA levels of GATA-3, IL-4, and IL-13 in dLNs (Fig 1, J). To increase relevance to human disease, we reproduced our experiments using house dust mite (HDM) antigen, a clinically important allergen (see Fig E9 in this article's Online Repository at www.jacionline.org). Similarly to the OVA-induced AAI experiments, AdSirt6 instillation ameliorated all the features of AAI induced by HDM significantly, confirming the protective effects of Sirt6 on AAI via modulation of T_H2 immune responses. Furthermore, AdmSirt6 instillation failed to exert a protective role in this study, suggesting that HDAC activity is crucial for downregulating T_H2 -mediated immune responses responsible for AAI.

GATA3 is known as a master transcription regulator driving T_H2 immune responses in asthma, and the importance of acetylation of GATA3 has been demonstrated in previous studies. Transgenic expression of hypoacetylated GATA3 leads to inhibition of T_H2 cytokine production and AAI in mice.^{5,6} However, the identity of the HDAC responsible for GATA3 deacetylation has not been determined. Here, we hypothesized that Sirt6 would deacetylate GATA3, thereby modulating its function as a master switch of T_H2 cell differentiation. To test our hypothesis, we first evaluated the acetylation status of GATA3 in lung tissues. As shown in Fig 2, A and B, AAI increased acetylation of GATA3 in lung tissues, whereas Sirt6 overexpression lowered it to the basal level, possibly through direct interaction. However, the catalytically inactive form of Sirt6 did not affect GATA3 acetylation. Chromatin immunoprecipitation results revealed decreased binding of GATA3 at the promoter region of IL-4 in Sirt6-overexpressing lung tissue (Fig 2, C). Further *in vitro* studies provided evidence of GATA3 deacetylation by Sirt6; Sirt6 overexpression led to hypoacetylation of GATA3 mediated by interaction between Sirt6 and GATA3, as elucidated by Co-IP (Fig 2, D and E). Colocalization of these proteins was visualized by confocal microscope analysis (Fig 2, F). To evaluate the functional consequences of Sirt6 overexpression, we overexpressed Sirt6 in a T-cell line (EL4) via adenovirus transduction followed by stimulation with phorbol 12-myristate 13-acetate and ionomycin. As shown in Fig 2, G and H, an increased level of Sirt6 in these cells was correlated with reduced secretion of T_H2 cytokines and downregulation of acetylated GATA3. However, haploinsufficiency of Sirt6 exerted opposite effects on T_H2 differentiation (Fig 2, I and J). These results indicate that Sirt6 deacetylates GATA3, leading to suppression of T_H2 immune responses.

Despite years of research, there is limited understanding of the immunological role of Sirt6, because most Sirt6 studies have focused on genomic stability, DNA repair, metabolism, and cancer. Some studies demonstrated an anti-inflammatory role of Sirt6 through modulation of macrophage function.⁷ Here, we demonstrate that overexpression of Sirt6, a class III HDAC, causes hypoacetylation of GATA-3 and negatively regulates T_H2 cell differentiation, leading to attenuation of AAI induced

by OVA or HDM. Our findings not only shed a light on the molecular mechanisms by which Sirt6 modulates T-cell differentiation but also provide evidence that enhanced HDAC activity via Sirt6 overexpression is closely related to a significant reduction in critical features of AAI induced by OVA and HDM. Therefore, targeting Sirt6 is a relevant approach that can be used to develop novel therapeutics for patients with asthma.

Methods are provided in this article's Online Repository at www.jacionline.org.

Hyun-Young Jang, PhD^a

Suna Gu, MS^b

Sang-Myeong Lee, DVM, PhD^b

Byung-Hyun Park, MD, PhD^a

From ^athe Department of Biochemistry, Chonbuk National University Medical School, Jeonju, Jeonbuk, Korea; and ^bthe Division of Biotechnology, College of Environmental and Bioresource Sciences, Chonbuk National University, Iksan, Jeonbuk, Korea. E-mail: bhpark@jbnu.ac.kr. Or: leesangm@jbnu.ac.kr.

This work was supported by the Basic Science Research Program (grant nos. 2015R1D1A1A01060441 and 2015R1D1A1A01057465) and by the Medical Research Center Program (2008-0062279) through the National Research Foundation funded by the Korean government.

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

REFERENCES

1. Cosio BG, Mann B, Ito K, Jazrawi E, Barnes PJ, Chung KF, et al. Histone acetylase and deacetylase activity in alveolar macrophages and blood mononocytes in asthma. *Am J Respir Crit Care Med* 2004;170:141-7.
2. Bhavsar P, Ahmad T, Adcock IM. The role of histone deacetylases in asthma and allergic diseases. *J Allergy Clin Immunol* 2008;121:580-4.
3. Aoshiba K, Nagai A. Differences in airway remodeling between asthma and chronic obstructive pulmonary disease. *Clin Rev Allergy Immunol* 2004;27:35-43.
4. Barnes PJ. The cytokine network in asthma and chronic obstructive pulmonary disease. *J Clin Invest* 2008;118:3546-56.
5. Yamagata T, Mitani K, Oda H, Suzuki T, Honda H, Asai T, et al. Acetylation of GATA-3 affects T-cell survival and homing to secondary lymphoid organs. *EMBO J* 2000;19:4676-87.
6. Zhu J, Min B, Hu-Li J, Watson CJ, Grinberg A, Wang Q, et al. Conditional deletion of Gata3 shows its essential function in T_H1 - T_H2 responses. *Nat Immunol* 2004;5:1157-65.
7. Kugel S, Mostoslavsky R. Chromatin and beyond: the multitasking roles for SIRT6. *Trends Biochem Sci* 2014;39:72-81.

<http://dx.doi.org/10.1016/j.jaci.2016.05.019>

METHODS

Animals

Pathogen-free male BALB/c mice and Sirt6^{+/-} mice were obtained from Samtaco (Osan, Korea) and Jackson Laboratory (Bar Harbor, Me), respectively. Sirt6^{+/-} mice were bred to generate Sirt6^{+/+} and Sirt6^{+/-}. The mice were 7 to 8 weeks old at the start of each experiment. All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH Publication No. 85-23, revised 2011). The current study protocol was also approved by the Institutional Animal Care and Use Committee of Chonbuk National University (Permit No. CBNU 2015-058).

Preparation of recombinant adenoviruses

Adenoviruses expressing Sirt6 (AdSirt6), catalytically inactive Sirt6-H133Y (AdmSirt6), Sirt1 (AdSirt1), and β -galactosidase (AdLacZ) were prepared as described previously.^{E1,E2}

Immunization and challenge

In the OVA-induced AAI model, mice were immunized intraperitoneally with 20 μ g of OVA (grade V; Sigma-Aldrich, St Louis, Mo) plus 2.25 mg aluminum hydroxide adjuvant on day 0 and with OVA alone on day 14. Immunized mice were exposed to aerosolized OVA (3%) on days 28, 29, and 30 using an ultrasonic nebulizer (NE-U12, Omron; output 0.8 mL/min) for 20 minutes in a Plexiglass exposure chamber (24.5 $\times$ 40.5 $\times$ 15.0 cm). The mice received a single intratracheal delivery of 30 μ L of saline or adenovirus (1×10^9 pfu) 3 days before the first OVA challenge. In the HDM-induced AAI model, male BALB/c mice were given 50 μ L HDM (25 μ g *Dermatophagoides pteronyssinus* extract, Greer Laboratories, Lenoir, NC) on days 0, 7, and 14 intranasally. On day 12, the mice received a single 30- μ L dose of saline or adenovirus (1×10^9 pfu) via intranasal delivery. Control mice group received 30 μ L of solvent aerosol (saline).

Bronchoalveolar lavage fluid

Mice were anesthetized, and 0.8 mL of PBS was administered intratracheally to mice through a flexible catheter. Bronchoalveolar lavage fluid (BALF) was collected while gently massaging the thorax. The recovered volume of PBS was approximately 0.6 mL. After centrifugation, cell pellets and supernatants were separated. The total cell number was determined using a hemocytometer under light microscope, and a cytospin preparation of BALF cells was stained with Diff-Quik (International Reagents Corp, Kobe, Japan). The different cell types were enumerated on the basis of morphology and staining profile. The supernatant was stored at -70°C until the cytokine assays were performed.

Cytokines analysis

Levels of IL-4 (eBioscience, San Diego, Calif), IL-13 (Invitrogen, Carlsbad, Calif), IL-5, and eotaxin (R&D Systems, Minneapolis, Minn) in BALF were determined by ELISA according to the manufacturers' protocols. Absorbance was recorded at 450 nm with a microplate reader. Amounts of cytokines in the samples were determined from a standard curve run on each plate.

Determination of airway hyperresponsiveness

Airway hyperresponsiveness to aerosolized methacholine (MCh) inhalation was assessed using whole-body plethysmography, as described elsewhere.^{E3} Briefly, mice were placed in a barometric plethysmographic chamber (All Medicus, Seoul, Korea), and baseline readings were taken and averaged for 3 minutes. Aerosolized MCh in increasing concentrations (0-50 mg/mL) was nebulized through an inlet of the main chamber for 3 minutes. Readings were taken and averaged for 3 minutes after each nebulization. Penh, which was calculated as (Expiratory time/Relaxation time - 1) $\times$ (Peak expiratory flow/Peak inspiratory flow), is a dimensionless value that represents a function of the proportion of maximal expiratory to maximal inspiratory box pressure signals and a function of the timing of expiration. Penh was used as a measure

of airway resistance to methacholine. Results were expressed for each MCh concentration as the percentage of baseline Penh values after saline exposure.

Measurement of OVA-specific IgE and IgG₁

To determine OVA-specific IgE or IgG₁ levels in serum, 96-well plates were coated with OVA overnight at 4°C at 100 μ g/well. OVA-coated plates were blocked with $1 \times$ assay diluent (eBioscience) for 1 hour at room temperature and washed with PBST (phosphate-buffered saline with Tween20). One hundred-fold dilutions of mouse serum were incubated in wells overnight at 4°C . Detection of OVA-specific IgE was carried out using biotinylated rat anti-mouse IgE (eBioscience), followed by avidin-horseradish peroxidase (HRP) and tetramethylbenzidine (TMB) as a substrate (eBioscience). Detection of OVA-specific IgG₁ was carried out using biotinylated rat antimouse IgG₁ (eBioscience), followed by avidin-HRP and TMB as a substrate (eBioscience).

RNA isolation and real-time RT-PCR

Total RNA was extracted from tissues using an RNA Iso kit (TaKaRa Bio, Kusatsu, Shiga, Japan). RNA was precipitated with isopropanol and dissolved in diethylpyrocarbonate-treated distilled water. First-strand cDNA was generated with oligoT-d adaptor primers by reverse transcriptase (TaKaRa). Specific primers were designed using qPrimerDepot (<http://mouseprimerdepot.nci.nih.gov>; Table E2). Real-time RT-PCR reactions comprised a final volume of 10 μ L, containing 10 ng of reverse-transcribed total RNA, 200 nM of forward and reverse primers, and PCR master mixture. RT-PCR was performed in 384-well plates using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, Calif). The relative expression of mRNA was calculated using the $\Delta\Delta\text{CT}$ threshold cycle method and glyceraldehyde 3-phosphate dehydrogenase as an internal control to normalize mRNA amount.

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation assay was performed using a chromatin immunoprecipitation assay kit (Millipore, Billerica, Mass) according to the manufacturer's instructions. Lung tissues (300 mg) were cross-linked by formaldehyde (1% final concentration) and incubated at room temperature for 20 minutes. The fixation reaction was stopped by adding glycine solution, and tissues were then washed with ice-cold PBS containing proteinase inhibitors. Samples were lysed for 10 minutes in lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris, pH 8.1) on ice, and chromatin was sheared by sonicating the samples 3 times for 15 seconds at maximum power with an ultrasonic processor, with 30 seconds of cooling on ice between each pulse. DNA-GATA3 complexes were immunoprecipitated with agarose beads and anti-GATA3 antibody (Thermo Scientific, Waltham, Mass) on a rolling shaker overnight at 4°C . Real-time RT-PCR was used to quantify the amount of precipitated IL-4 promoter DNA. As a loading control, PCR was performed using input DNA purified from chromatin before immunoprecipitation. Data were normalized to the corresponding input DNA values and expressed as a percentage of the input. Primers used to amplify the IL-4 promoter were 5'-ACCTGTGACCTCTTCTCTCTCT-3' and 5'-CCAATTGGAAATGAAACACCCC-3'.

Cell surface staining and intracellular cytokine staining for flow cytometry

Single-cell suspensions prepared from dLNs were stained with anti-CD4-PerCP/Cy5.5 and anti-CD69-fluorescein isothiocyanate antibodies (eBioscience) for 30 minutes on ice and washed with Fluorescence Activated Cell Sorting (FACS) buffer (2% FBS in PBS) 3 times. Then, cells were analyzed using an Accuri flow cytometer (BD Biosciences, San Jose, Calif). Cells were first stained for cell surface CD4 molecules with anti-CD4-PerCP/Cy5.5 antibody and stimulated with 50 ng/mL phorbol 12-myristate 13-acetate and 1 μ M ionomycin in the presence of brefeldin A (eBioscience) for 4 hours. Cells were fixed with 4% paraformaldehyde solution and permeabilized with $1 \times$ permeabilization solution (eBioscience), followed by staining with anti-IL-4-PE antibody. Stained cells were evaluated by flow cytometry analysis (Accuri, BD Biosciences). All antibodies were purchased from eBioscience or Biolegend (San Diego, Calif).

Confocal microscopy

HEK293T cells cultured on coverslips in 6-well plates were transfected with HA-Sirt6 or Flag-GATA3 plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Cells were washed twice with cold PBS for 5 minutes, fixed with 2% paraformaldehyde for 40 minutes, and then permeabilized with 0.1% Triton X-100 for 40 minutes. Cells were then blocked with PBS containing 5% NGS for 30 minutes and incubated with anti-Flag mouse antibody for GATA3 (1:100) and anti-HA rabbit antibody for Sirt6 (1:100) overnight. After washing with PBS containing 0.1% Triton X-100, cells were stained with fluorescein isothiocyanate-conjugated anti-rabbit IgG antibody (1:100) and Alexa568-conjugated goat anti-mouse IgG antibody for 40 minutes. Coverslips were washed extensively with PBS and stained with 4'-6-diamidino-2-phenylindole, dihydrochloride (Invitrogen) for 5 minutes. Last, coverslips were mounted onto glass slides with Vectashield hardset antifade mounting medium (Vector Laboratories, Burlingame, Calif). Images were acquired with an LSM510 confocal laser scanning microscope (Carl Zeiss, Ostalbkreis, Germany).

Western blotting

Tissues and cells were homogenized in Tissue Protein Extraction Reagent or Mammalian Protein Extraction Reagent (Thermo Scientific). Tissue or cell lysates containing 10 μ g of total protein were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. After blocking with 5% skim milk in Tris-buffered saline with Tween20 (TBST), blots were probed with appropriate primary antibodies in 5% BSA in TRIS-buffered saline overnight at 4°C. Then, blots were washed with TBST and incubated with HRP-conjugated secondary antibodies (Thermo Scientific) for 2 hours at room temperature. After washing 3 times with TBST, blots were developed with Supersignal West Femto Maximum Sensitivity substrate (Thermo Scientific) and analyzed with a Las-4000 imaging system (GE Healthcare Life Science, Pittsburgh, Pa). The following antibodies were purchased from the indicated sources: Sirt1 (SIR11), Sirt6 (Abcam, Cambridge Science Park, Cambridge, United Kingdom), HA (Y-11), Sirt2 (Santa Cruz Biotechnology, Dallas, Tex), Sirt4 and Sirt7 (Biovision, Milpitas, Calif), Sirt3 (D22A3), acetylated-lysine (Cell Signaling Technology, Beverly, Mass), Sirt5 (LS Bio, Seattle, Wash), β -actin (AC-15), Flag (M2) (Sigma-Aldrich), GATA3 (1A12-1D9) (Thermo Scientific), and glyceraldehyde 3-phosphate dehydrogenase (A531) (Bioworld Technology, St Louis Park, Minn).

Immunoprecipitation analyses

Tissue lysates or cell lysates were precleared with protein G-agarose (Invitrogen) and incubated with the indicated antibodies at 4°C overnight. Protein G-agarose was then added to the lysates followed by a 2-hour incubation at 4°C. Immunoprecipitates were subjected to western blot analysis.

Histology

Lungs were fixed with 10% formalin, and the tissues were embedded in paraffin. Fixed tissues were cut at a thickness of 4 μ m, placed on glass slides, and deparaffinized. Sections were stained with H&E dye for light microscopy evaluation. The degree of peribronchial and perivascular inflammation was evaluated on a subjective scale of 0 to 3, as described elsewhere.^{E4} A value of 0 indicated no detectable inflammation, a value of 1 indicated occasional cuffing with inflammatory cells, a value of 2 was assigned if most bronchi or vessels were surrounded by a thin layer (1-5 cells) of inflammatory cells, and a value of 3 was assigned when most bronchi or vessels were surrounded by a thick layer (>5 cells) of inflammatory cells. To detect mucin, tissues were stained with periodic acid-Schiff (PAS) dye and counterstained with hematoxylin. The number of PAS-positive or PAS-negative airway epithelial cells was assessed in 5 randomly selected medium-sized bronchi (defined by having approximately 100-150 luminal airway epithelial cells). The percentage of PAS-positive airway epithelial cells was then calculated for each airway and the mean was determined for each mouse.

EL4 cell transduction with adenovirus

Murine T-cell lymphoma EL4 cells were purchased from the American Type Culture Collection (Manassas, Va) and cultured in Dulbecco modified

Eagle medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 μ g/mL streptomycin. EL4 cells were seeded at a concentration of 1×10^6 /mL. Adenovirus was added directly to the cells and mixed by swirling plates. Six hours after transduction, cells were treated with cell stimulation cocktail (eBioscience). After 48 hours, IL-4 concentration in the culture supernatants was measured by ELISA, and cell lysates were used in immunoprecipitation experiments.

CD4⁺ T-cell preparation

Single-cell suspensions were prepared from lymph nodes and spleens from 2 to 3 mice. CD4⁺ T cells were isolated using the Dynabeads FlowComp mouse CD4⁺ T-cell isolation kit (Invitrogen) in accordance with the manufacturer's instructions. Purity of CD4⁺ T-cell populations was approximately 95%, as assessed by flow cytometry (Accuri flow cytometer, BD Biosciences).

T_H2 differentiation

CD4⁺ T cells were activated with plate-bound anti-CD3 antibody (1 μ g/mL) and soluble anti-CD28 antibody (2 μ g/mL). Cells were cultured under T_H2 (10 ng/mL IL-4, 1 μ g/mL anti-IL-12, and 5 μ g/mL anti-IFN- γ antibody) polarization conditions in 2 mL RPMI complete media. Four days later, cells were stimulated with phorbol 12-myristate 13-acetate and ionomycin in the presence of brefeldin A (eBioscience) for 4 hours and intracellular cytokine staining was performed. All cytokines and recombinant proteins were purchased from eBioscience.

Acetylated GATA3 ELISA

GATA-3 acetylation levels were determined by sandwich ELISA as described in a previous study with some modifications.^{E5} In brief, 96-well ELISA plates (Thermo Scientific) were coated with anti-GATA-3 antibody (1 μ g/mL) in NaHCO₃ coating buffer overnight at 4°C. After blocking with blocking buffer (eBioscience), plates were washed with washing buffer (0.2% Tween 20/PBS). Nuclear proteins were added into the plates in duplicate followed by a 2-hour incubation at room temperature. Plates were then washed 5 times with washing buffer, after which antiacetylated rabbit antibody (1 μ g/mL) was added to each well. After incubation at room temperature for 2 hours, plates were washed 5 times with washing buffer, followed by addition of antirabbit IgG-HRP (Pierce, 1:1000 dilution). Plates were then incubated for an additional 1 hour at room temperature followed by 5 washes using wash buffer. TMB substrate (eBioscience) was added and the reaction was stopped by adding 1 N H₂SO₄. Absorbance was recorded at 450 nm after 10 to 15 minutes using a microplate reader (Molecular Devices, Palo Alto, Calif).

Statistical analysis

Data are expressed as means $\pm$ SEMs. Statistical comparisons were performed using 1-way ANOVA followed by the Tukey test. In some experiments, student *t* test was performed to detect between-group differences. A *P* value of less than .05 was accepted as an indication of statistical significance.

REFERENCES

- E1. Lee HS, Ka SO, Lee SM, Lee SI, Park JW, Park BH. Overexpression of sirtuin 6 suppresses inflammatory responses and bone destruction in mice with collagen-induced arthritis. *Arthritis Rheum* 2013;65:1776-85.
- E2. Lee JH, Song MY, Song EK, Kim EK, Moon WS, Han MK, et al. Overexpression of SIRT1 protects pancreatic β -cells against cytokine toxicity by suppressing the nuclear factor- κ B signaling pathway. *Diabetes* 2009;58:344-51.
- E3. Kim SR, Kim DI, Kang MR, Lee KS, Park SY, Jeong JS, et al. Endoplasmic reticulum stress influences bronchial asthma pathogenesis by modulating nuclear factor κ B activation. *J Allergy Clin Immunol* 2013;132:1397-408.
- E4. Tournoy KG, Kips JC, Schou C, Pauwels RA. Airway eosinophilia is not a requirement for allergen-induced airway hyperresponsiveness. *Clin Exp Allergy* 2000;30:79-85.
- E5. Colley T, Mercado N, Kunori Y, Brightling C, Bhavsar PK, Barnes PJ, et al. Defective sirtuin-1 increases IL-4 expression through acetylation of GATA-3 in patients with severe asthma. *J Allergy Clin Immunol* 2016;137:1595-7.e7.

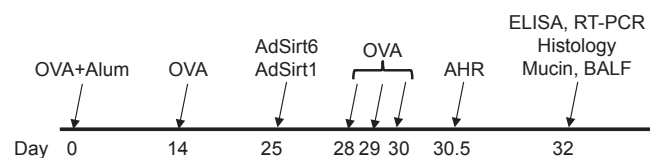


FIG E1. Schematic diagram of the experimental protocol. Mice were immunized intraperitoneally with 20 μ g of OVA plus 2.25 mg aluminum hydroxide adjuvant on day 0 and with OVA alone on day 14. On day 25, mice received a single 30- μ L dose of saline or adenovirus (1×10^9 pfu) via intratracheal delivery. Immunized mice were exposed to aerosolized OVA on days 28, 29, and 30. Mice were euthanized, and peripheral blood, BALF, dLN, and lung tissues were prepared for analysis at the indicated time points. *AHR*, Airway hyperresponsiveness.

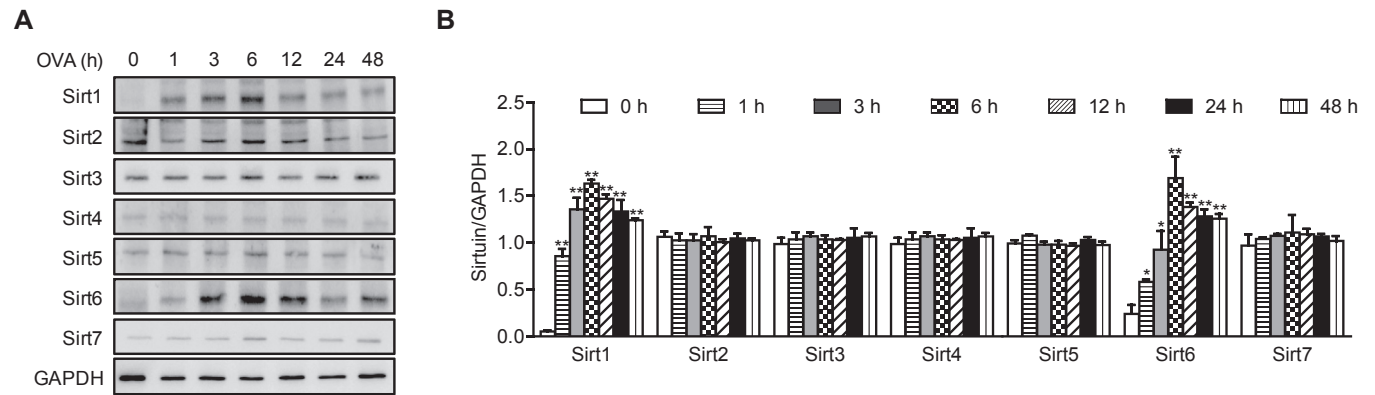


FIG E2. Sirtuin expression in lung tissues after OVA immunization and challenge. **A**, After the last OVA challenge, lung tissues were collected at the indicated time points. Protein levels of sirtuins in lung tissues were analyzed by western blotting. **B**, Band densities were analyzed and are shown as means $\pm$ SEMs ($n = 4$). *GAPDH*, Glyceraldehyde 3-phosphate dehydrogenase. * $P < .05$ and ** $P < .01$ versus 0 hour.

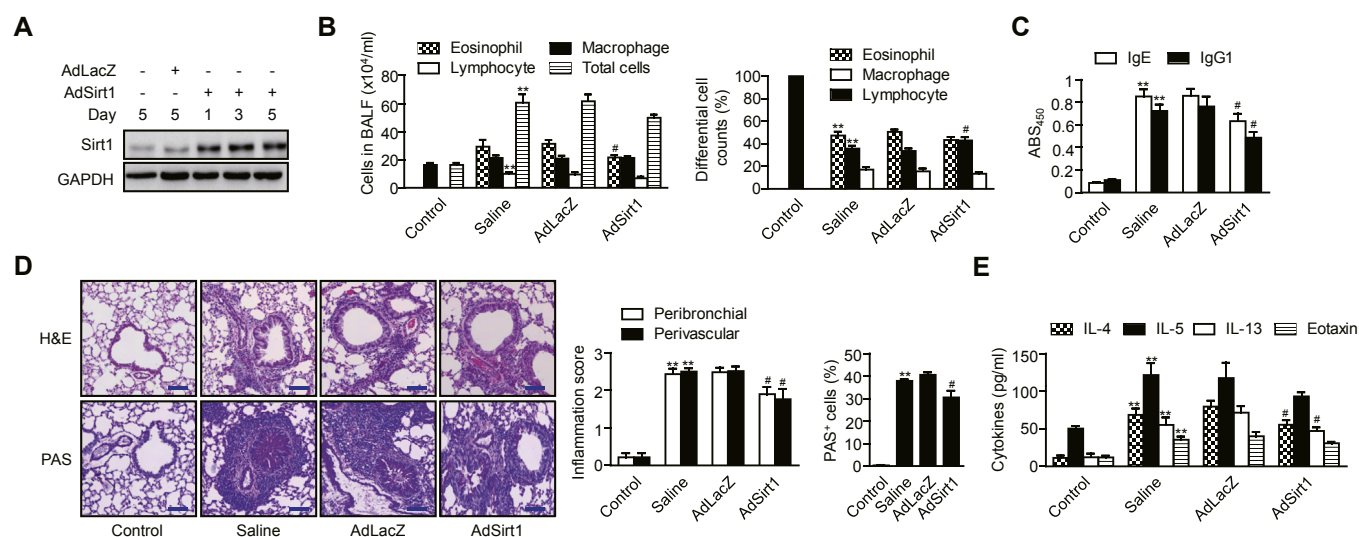


FIG E3. Effects of Sirt1 overexpression on AAI induced by OVA immunization and challenges. **A**, Mice were instilled with 1×10^9 pfu of either AdLacZ or AdSirt1 intratracheally. Lung homogenates prepared at 1, 3, and 5 days after viral instillation were subjected to western blot analysis with anti-Sirt1 antibody. **B**, Total and differential cell counts in BALF were analyzed ($n = 6$). **C**, OVA-specific IgE or IgG₁ levels in serum were determined using ELISA ($n = 6$). **D**, Paraffin-embedded lung sections were stained with H&E, and perivascular and peribronchovascular inflammation was scored as described in the [Methods](#) section ($n = 6$). PAS staining was performed, and mucin within the airway stained pink or red. PAS-positive cells were numerated under a light microscope ($n = 6$). Bar = 250 μ m. **E**, Various cytokines in BALF were measured by specific ELISAs. GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; H&E, hematoxylin and eosin. Values are means $\pm$ SEMs. ** $P < .01$ versus control; # $P < .05$ versus AdLacZ.

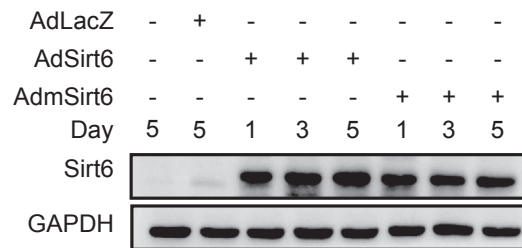


FIG E4. Sirt6 expression in lung tissues after virus instillation. Mice were instilled with 1×10^9 pfu of AdLacZ, AdSirt6, or AdmSirt6 intratracheally. Lung homogenates prepared at 1, 3, and 5 days after viral instillation were subjected to western blot analysis with anti-Sirt6 antibody. This experiment was repeated more than 3 times; a representative result is shown. *GAPDH*, Glyceraldehyde 3-phosphate dehydrogenase.

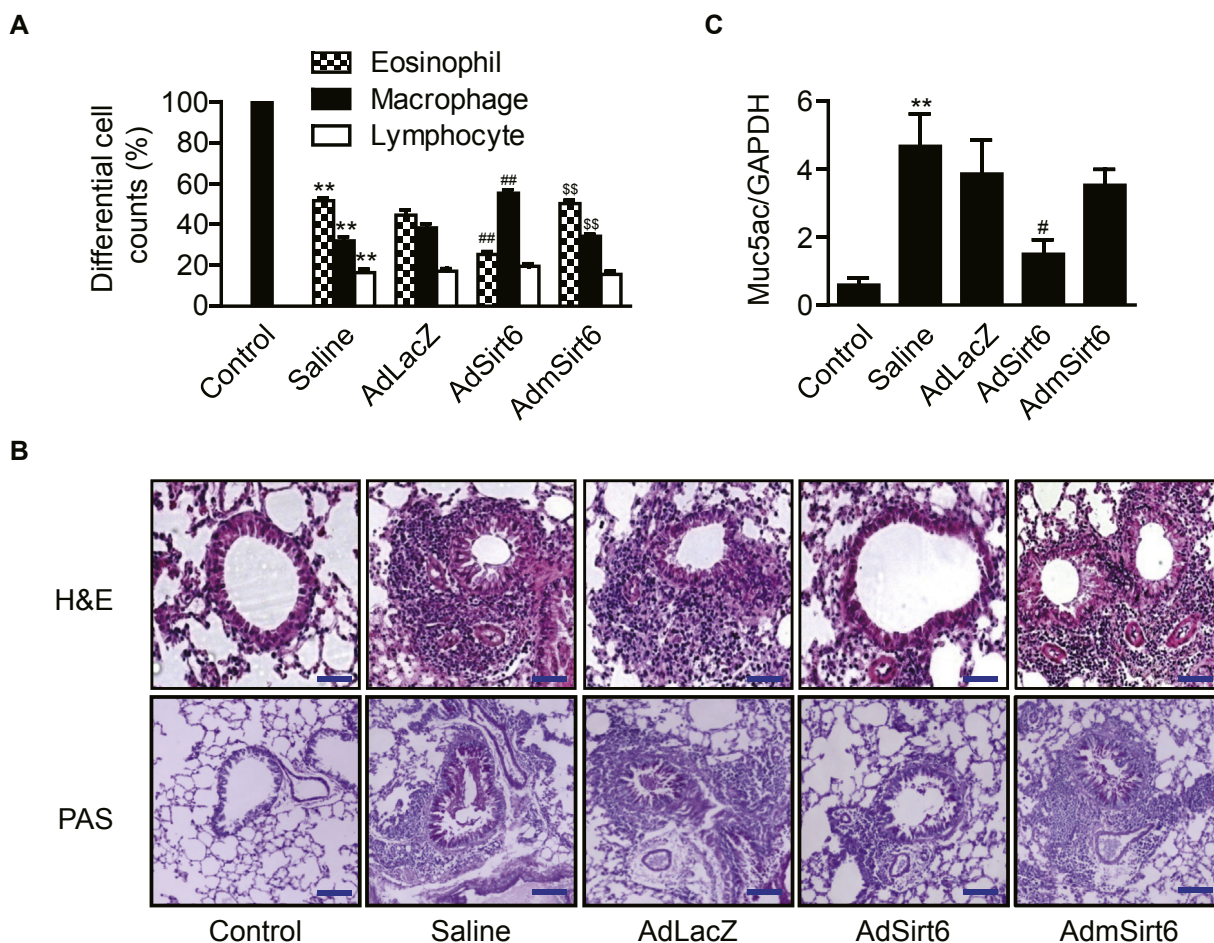


FIG E5. Effects of Sirt6 overexpression on BALF cells, inflammatory cells infiltration into lung tissues, and mucin production. **A**, Proportion of eosinophils, macrophages, and lymphocytes in BALF was analyzed ($n = 13$). **B**, Paraffin-embedded lung sections were stained with H&E (100 $\times$). Bar = 250 μ m. PAS staining was performed using lung tissue sections, and mucin within the airway stained *pink* or *red*. Bar = 250 μ m. Representative photos are shown. **C**, mRNA level of Muc5ac was analyzed by real-time RT-PCR ($n = 5$). GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; H&E, hematoxylin and eosin. Values are means $\pm$ SEMs. ** $P < .01$ versus control; # $P < .05$ and ## $P < .01$ versus AdLacZ; \$\$ $P < .01$ versus AdSirt6.

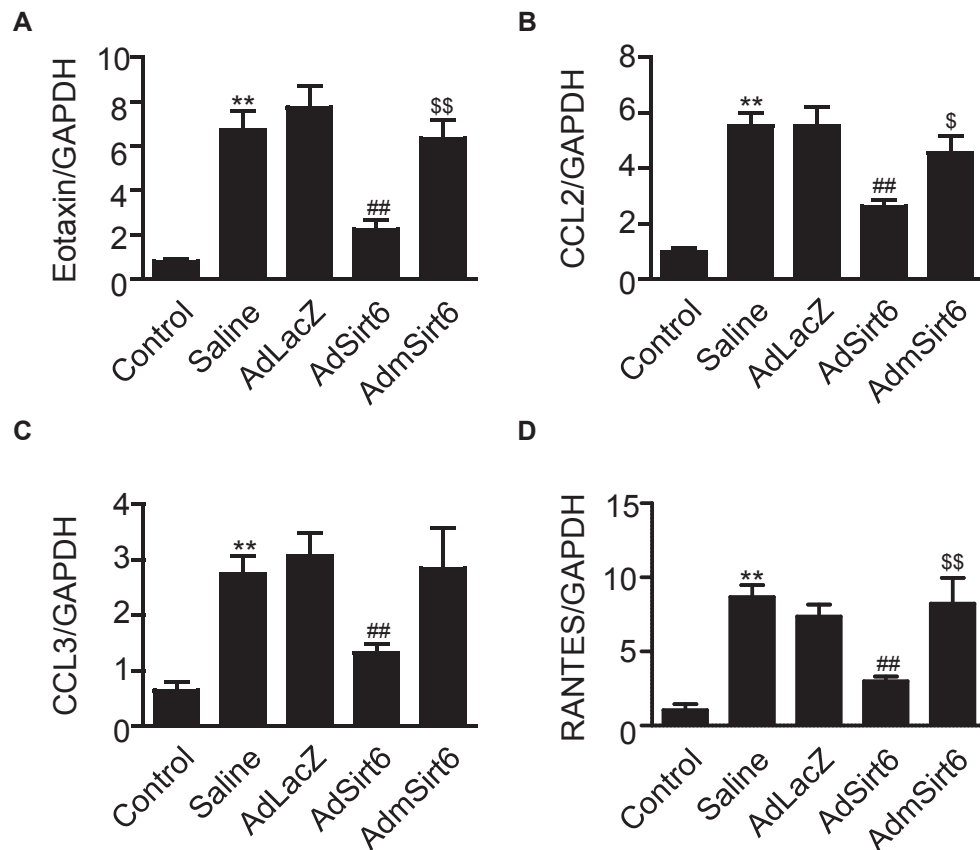


FIG E6. Effects of Sirt6 overexpression on chemokine production in lung tissues. Total RNA was extracted from lung tissues and mRNA levels of eotaxin (**A**), CCL2 (**B**), CCL3 (**C**), and RANTES (**D**) were analyzed by real-time RT-PCR. *GAPDH*, Glyceraldehyde 3-phosphate dehydrogenase. Values are the mean $\pm$ SEM ($n = 7$). ** $P < .01$ versus control; ## $P < .01$ versus AdLacZ; \$ $P < .05$ and \$\$ $P < .01$ versus AdSirt6.

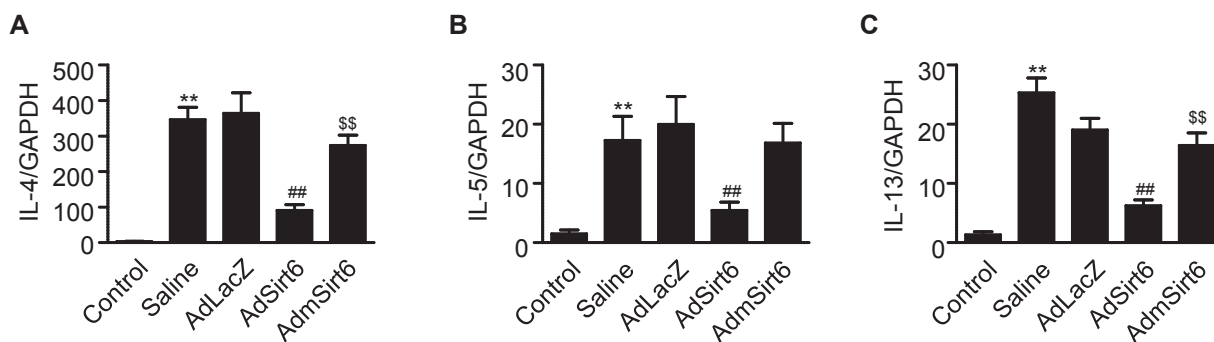


FIG E7. mRNA levels of T_H2 cytokines in lung tissues. Total RNA was extracted from lung tissues and mRNA levels of IL-4 (**A**), IL-5 (**B**), and IL-13 (**C**) were analyzed by real-time RT-PCR. *GAPDH*, Glyceraldehyde 3-phosphate dehydrogenase. Values are means $\pm$ SEMs (n = 7). ** P < .01 versus control; ## P < .01 versus AdLacZ; \$\$ P < .01 versus AdSirt6.

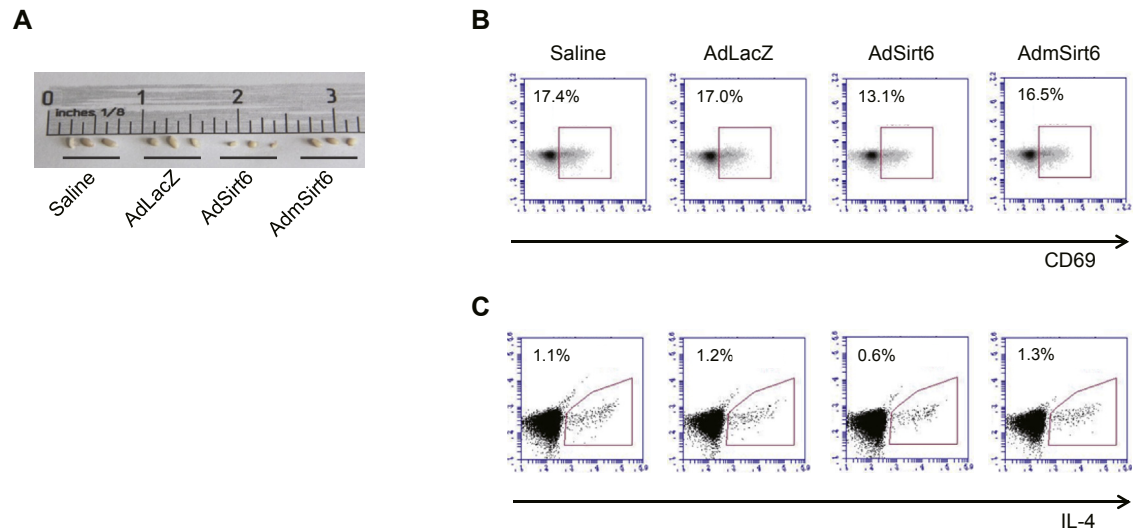


FIG E8. Effects of Sirt6 overexpression on CD4⁺ T-cell activation and T_H2 cell differentiation in dLNs. **A**, Photographs of dLNs. dLNs were collected 2 days after the last OVA challenge, and single-cell suspensions were prepared. CD4⁺ T cells were analyzed for CD69 (**B**) and IL-4 expression (**C**) by flow cytometry.

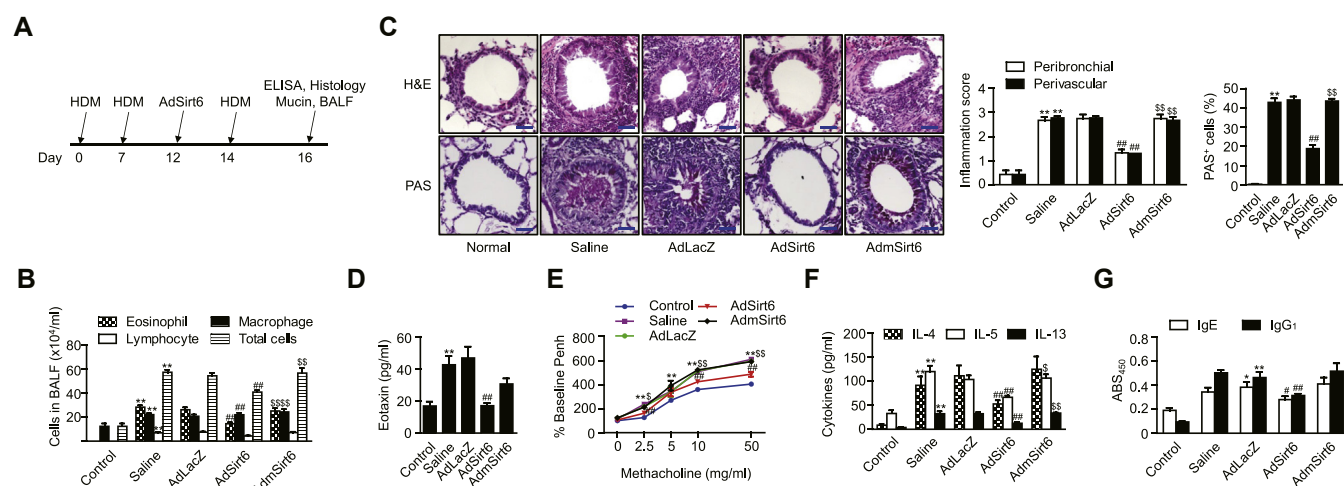


FIG E9. Effects of Sirt6 overexpression on AAI in response to HDM. **A**, Schematic representation of the experimental protocol using intranasal administration of HDM and intranasal instillation of adenoviruses. **B**, Differential cell counts in BALF were analyzed 48 hours after the HDM challenge; the absolute number of each cell type was counted ($n = 8$). **C**, Paraffin-embedded lung sections were stained with H&E, and peribronchial and perivascular inflammation was scored as described in the [Methods](#) section. PAS staining was performed, and mucin within the airway stained a pink to red color. PAS-positive cells were numerated under light microscope ($n = 12$). Bar = 250 μ m. **D**, Eotaxin levels in BALF were evaluated using ELISA. **E**, AHR was assessed as described in the [Methods](#) section. All animals were nebulized with various concentrations of methacholine as a bronchoconstrictor. Data are shown as percent increase in Penh relative to baseline ($n = 4$). **F**, Cytokine levels in BALF were measured using ELISA ($n = 6$). **G**, HDM-specific IgE or IgG₁ levels in serum and cytokines in BALF were determined using ELISA ($n = 6$). Values are means $\pm$ SEM. AHR, Airway hyperresponsiveness; H&E, hematoxylin and eosin. * $P < .05$ and ** $P < .01$ versus control; # $P < .05$ and ## $P < .01$ versus AdLacZ; \$ $P < .05$ and \$\$ $P < .01$ versus AdSirt6.

TABLE E1. Differential cell counts of dLNs

Cell surface marker	Saline	AdLacZ	AdSirt6	AdmSirt6
CD4 ⁺ (%)	43.66	43.42	36.24*	42.42†
CD8 ⁺ (%)	22.75	23.95	18.61*	22.73†
B220 ⁺ (%)	46.32	48.59	44.77	44.14
CD11c ⁺ (%)	3.10	4.03	3.12	3.72
CD11b ⁺ (%)	1.88	2.00	1.52	2.04

Values are means $\pm$ SEMs (n = 8).

**P* < .01 versus AdLacZ.

†*P* < .01 versus AdSirt6.

TABLE E2. Sequences and accession numbers of primers (forward, FOR; reverse, REV) used in real-time RT-PCR

Gene	Primer sequences	Genbank accession no.
IL-4	FOR: TCGGCATTTTGAACGAGGTC REV: GAAAAGCCCGAAAGAGTCTC	NM_021283
IL-5	FOR: TCACCGAGCTCTGTTGACAA REV: CCACACTTCTCTTTTGGCG	NM_010558
IL-13	FOR: CGCAAGGCCCCCACTAC REV: AAAGTGGGCTACTTCGATTTTGG	NM_008355
Eotaxin	FOR: TTGGCTTTGCAGCTCTTCCT REV: TGACTGTGCCGTGGCAGTA	NM_008337
Muc5ac	FOR: AAGCCTGCAATGAAGGAGAA REV: CTGCAGTCCAGGGAAGAAAG	NM_008337
GATA-3	FOR: GGAAACTCCGTCAGGGCTA REV: AGAGATCCGTGCAGCAGAG	NM_008091
RANTES	FOR: TTTGCCTACATTGCCCGC REV: TTTTCGGGTGACAAAGACGACT	NM_013653
CCL2	FOR: ATTGGGATCATCTTGCTGGT REV: CCTGCTGTTACAGTTGCC	NM_011333
CCL3	FOR: GTGGAATCTTCCGGCTGTAG REV: ACCATGACACTCTGCAACCA	NM_011337
GAPDH	FOR: CGTCCCGTAGACAAAATGGT REV: TTGATGGCAACAATCTCCAC	NM_008084

GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.