

Letter to the Editor

Defective sirtuin-1 increases IL-4 expression through acetylation of GATA-3 in patients with severe asthma

To the Editor:

Patients with severe asthma are poorly responsive to currently available asthma therapies, and a high level of T_H2 development has been observed.¹ GATA-3, a T_H2 transcription factor that is increased in the airways of asthmatic patients, is known to be an acetylated protein,² suggesting that protein deacetylases, such as sirtuin-1 (SIRT1), a class III histone deacetylase, might regulate its function.

In the first cohort SIRT1's deacetylase activity was shown to be decreased in PBMCs from patients with severe asthma when compared with those of patients with nonsevere asthma and healthy subjects (Fig 1, A, and see Table E1 in this article's Online Repository at www.jacionline.org). Decreased SIRT1 activity was also positively correlated with decreased FEV₁ (Fig 1, B). Because the expression of SIRT1 mRNA transcripts quantified by using quantitative RT-PCR (qRT-PCR) showed no change

between groups, the reduction of SIRT1 is not due to reduced gene transcription (Fig 1, C). In the second cohort (see Table E2 in this article's Online Repository at www.jacionline.org) we found that SIRT1 protein expression in whole-cell extracts from PBMCs was significantly reduced in patients with severe asthma when compared with that of healthy subjects (Fig 1, D and E), although no decrease was observed in other sirtuin members (SIRT2, SIRT3, SIRT6, and SIRT7; Fig 1, E, and see Fig E1, A-D, in this article's Online Repository at www.jacionline.org). To date, reduced SIRT1 expression has been associated with oxidative stress through cigarette smoke or hydrogen peroxide exposure and a high-fat diet.³ Because oxidative stress has been strongly linked to the pathogenesis of severe asthma,⁴ the reduction of SIRT1 quite possibly has its origin in this increased oxidative condition. In addition, c-Jun N-terminal kinase (JNK) 2 is reported to stabilize SIRT1 protein through phosphorylation,⁵ but JNK phosphorylation is reduced in the airways of asthmatic patients.⁶ Thus a defect of JNK in asthmatic patients might cause reduction of SIRT1 protein levels.

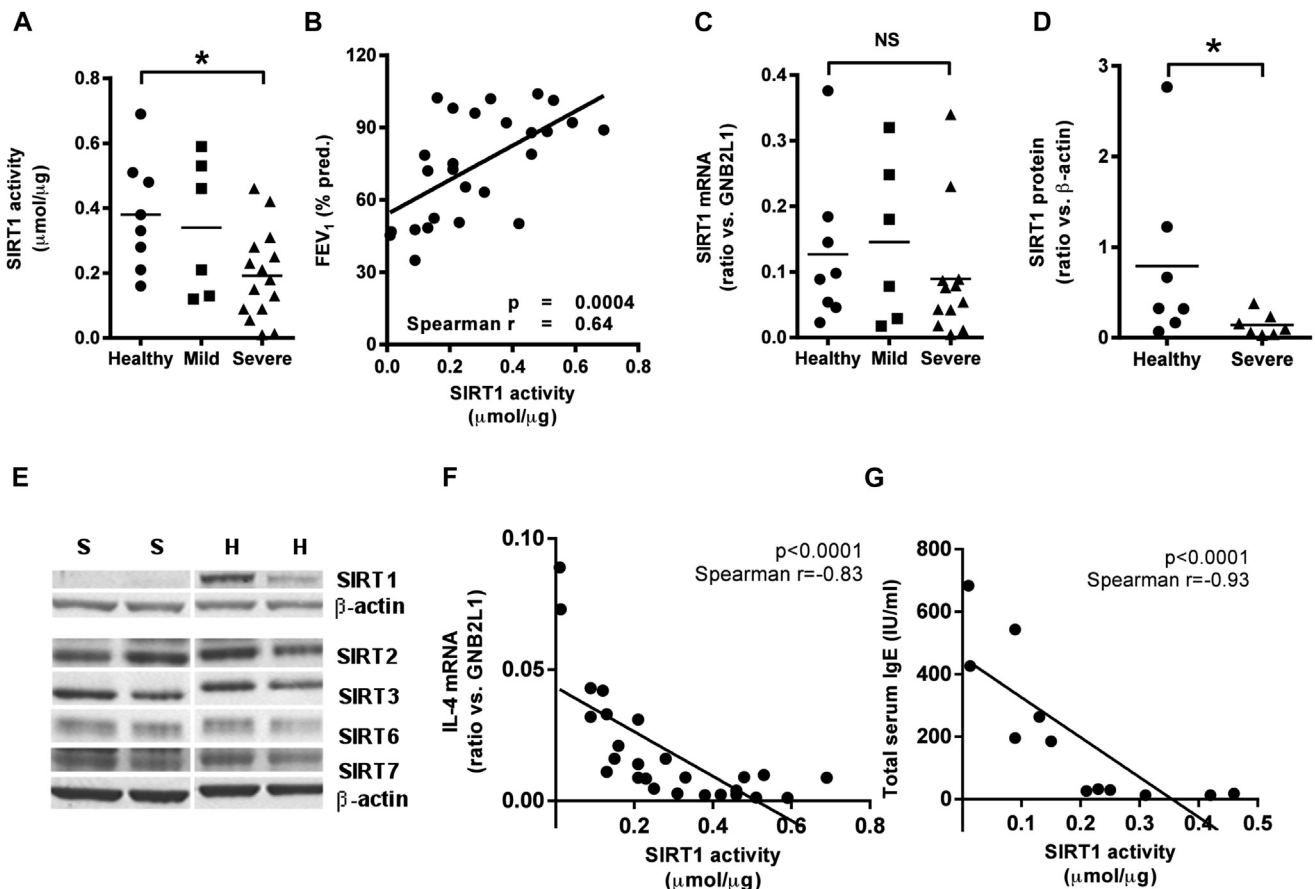


FIG 1. SIRT1 in PBMCs from healthy subjects ($n = 8$), patients with mild asthma ($n = 6$), and patients with severe asthma ($n = 15$). **A**, SIRT1 activity. **B**, Spearman nonparametric correlation analysis of FEV₁ (percent predicted) data and SIRT1 activity. **C**, SIRT1 mRNA. **D**, SIRT1 protein expression (7 healthy subjects and 8 patients with severe asthma). **E**, representative Western blots of sirtuin expression (healthy subjects [H] and patients with severe asthma [S]). **F** and **G**, Spearman nonparametric correlation analysis of SIRT1 activity with IL-4 mRNA expression (Fig 1, F) and total serum IgE levels (Fig 1, G). * $P < .05$. NS, Not significant.

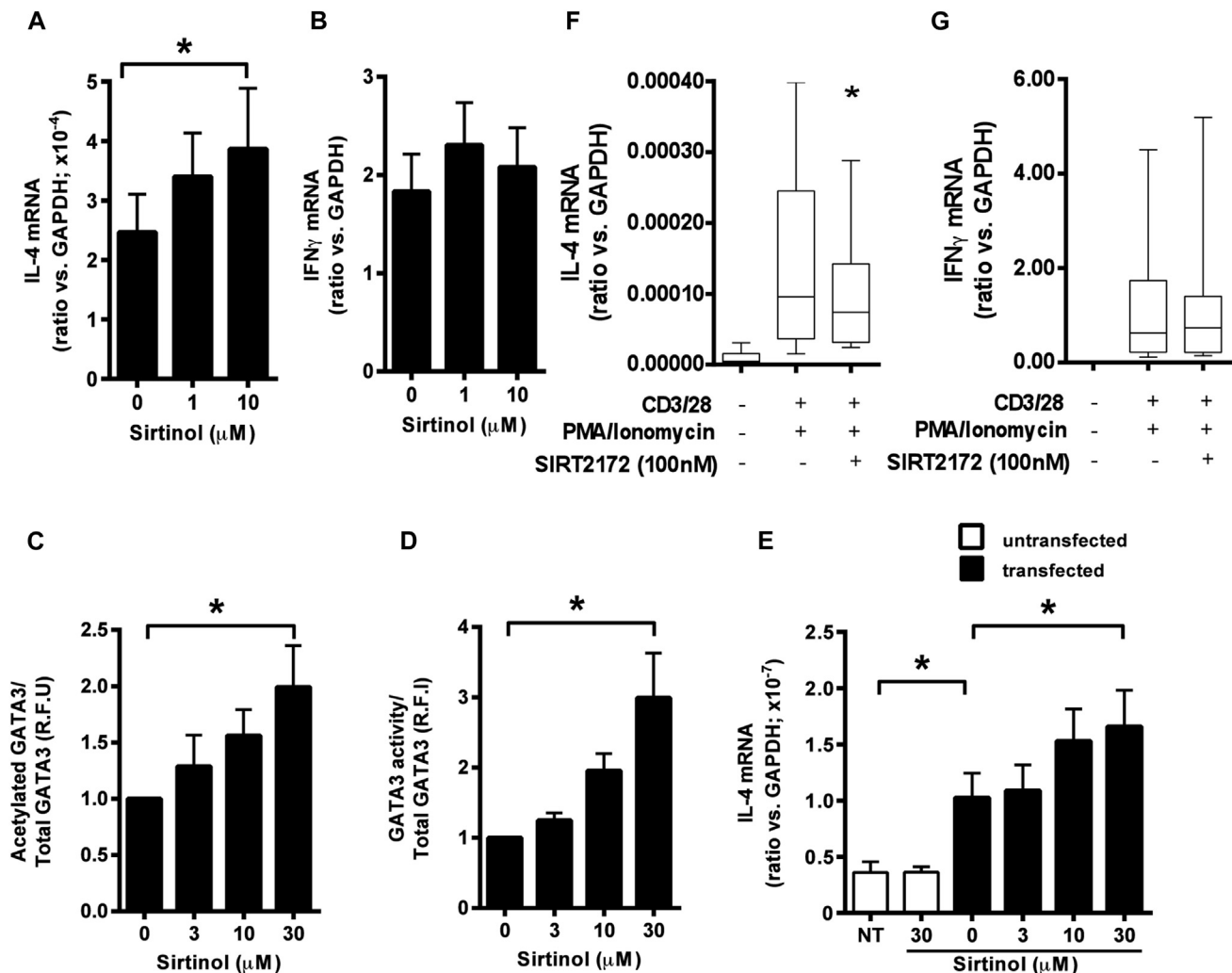


FIG 2. Inhibition of sirtuins promotes a T_H2 phenotype and increases acetylation of GATA-3. **A** and **B**, IL-4 and IFN- γ mRNA expression in PBMCs ($n = 3$). **C**, Acetylation of GATA-3 in HUT78 nuclear protein extract ($n = 6$). **D**, GATA-3 activity measured by using GATA-3 DNA binding and normalized against total GATA-3 protein ($n = 5$). **E**, IL-4 mRNA expression in untransfected or stably GATA-3 transfected HEK293 cells ($n = 4$). NT, Nontreatment. **F** and **G**, Effects of a SIRT1 activator (SIRT2172) on IL-4 and IFN- γ expression in PBMCs from patients with severe asthma. * $P < .05$. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

Analysis of biomarkers demonstrated a strong negative correlation between SIRT1 activity and expression of IL-4 transcripts in PBMCs (Fig 1, F), as well as total serum IgE levels (Fig 1, G). In addition, there was a significant reduction of SIRT1 activity with an increase in IL-4 transcripts in patients with atopic severe asthma compared with those with nonatopic severe asthma in the first cohort (see Fig E1, E and F). A semiquantitative cytokine array analysis in PBMCs from healthy subjects indicated that the sirtuin inhibitor sirtinol upregulated the expression of T_H2 -associated cytokines but had no effect on the expression of T_H1 cytokines (see Fig E2 in this article's Online Repository at www.jacionline.org). Further analysis using real-time quantitative PCR revealed that sirtinol at 10 μ mol/L significantly ($P < .05$) increased the expression of IL-4 and IL-13 when healthy PBMCs were activated by anti-CD3, anti-CD28, phorbol 12-myristate 13-acetate (PMA), and ionomycin (Fig 2, A, and see Fig E3, A, in this article's Online Repository at www.jacionline.org).

although we could not use sirtinol at 30 μ mol/L or greater because of a decrease in cell numbers (data not shown). This was also confirmed by using specific SIRT1/2 inhibition by cambinol at 50 μ mol/L (see Fig E3, B and C). Conversely, mRNA expression of the T_H1 cytokine IFN- γ showed no change with either inhibitor (Fig 2, B, and see Fig E3, D). Intracellular cytokine analysis by using flow cytometry also revealed that sirtinol showed a trend toward increased intracellular IL-4 levels but did not alter intracellular IFN- γ levels in $CD4^+$ cells separated from PBMCs of healthy subjects (see Fig E3, E and F). According to additional analysis with $CD4^+CD45RA^+$ T cells from cord blood, which were induced to differentiate into T_H1 and T_H2 subsets, T_H1 -dominant cells preincubated with sirtinol demonstrated no significant change in the ratios of IFN- γ to IL-4 and T-box transcription factor (T-bet) to GATA-3 when normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH; data not shown). However, there was a highly

significant increase in the ratio of IL-4 to IFN- γ in T_H2-dominant cells (see Fig E3, G). This was accompanied by a borderline significant ($P = .06$) increase in the ratio of GATA-3 to T-bet (see Fig E3, H). In addition, we have preliminary data that indicate sirtinol promotes T_H2-specific proliferation after 21 days of culture (data not shown). This is the first *in vitro* evidence in human cells that inhibition of SIRT1 promotes a T_H2-like phenotype in T cells.

The effect of sirtinol on the acetylation of GATA-3 was examined to identify the mechanism of increase in T_H2 cytokine expression by sirtuin inhibition. Sirtuin inhibition by 30 μ mol/L sirtinol resulted in increased acetylation of GATA-3 in the nuclear protein of HUT78 T lymphocytes (Fig 2, C) without any increase in GATA-3 protein expression (data not shown). In addition, immunoprecipitated GATA-3 was confirmed to be highly acetylated when sirtuins were inhibited (see Fig E3, I). Binding of GATA-3 to its response element was also enhanced when treated with sirtinol at 30 μ mol/L, suggesting that increased acetylation of GATA-3 directly affected its activity (Fig 2, D). In addition, GATA-3 overexpression increased IL-4 mRNA expression in HEK293 cells, which are largely GATA-3 deficient, and sirtuin inhibition induced IL-4 gene expression further (Fig 2, E). These data indicate that T_H2 development in human subjects is reliant on a relationship between SIRT1 and GATA-3. However, in this study we were unable to show a statistically significant correlation between IL-5 transcripts and SIRT1 activity in all subjects, as well as between SIRT1 activity and blood eosinophils (as a percentage) in patients with severe asthma (see Fig E1, G and H). GATA-3 is essential for initial expression of T_H2 cytokines, such as IL-4 and IL-5, but less important for the maintenance of IL-4 expression in mature cells.⁷ Therefore other T_H2 transcription factors, such as signal transducer and activator of transcription 6, c-Maf, or nuclear factor of activated T cells, might be involved. It is also possible that acetylated GATA-3 preferentially associates with the IL-4 promoter. Even more importantly, a SIRT1 activator (SRT2172)⁸ significantly reduced IL-4 mRNA expression in PBMCs from 10 patients with severe asthma without any significant change in IFN- γ expression (Fig 2, F and G). This supports the case that reduced SIRT1 activity causes increased IL-4 expression in patients with severe asthma.

Thus we demonstrate that SIRT1 plays a role in maintaining the T_H cell balance and preventing sustained cellular differentiation toward a T_H2 phenotype. This article provides new evidence that reduction in SIRT1 levels in patients with severe asthma contributes to increased IL-4 expression and that this would be a novel target for the treatment of severe asthma.

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Thomas Colley, PhD^a
Nicolas Mercado, PhD^a
Yuichi Kunori, PhD^a
Christopher Brightling, FRCP^b
Pankaj K. Bhavsar, PhD^a
Peter J. Barnes, FRS^a
Kazuhiro Ito, PhD^a

From ^athe Airway Disease Section, National Heart and Lung Institute, Imperial College London, London, United Kingdom, and ^bRespiratory Medicine, Glenfield Hospital, University of Leicester, Leicester, United Kingdom. E-mail: k.ito@imperial.ac.uk.

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METHODS

Patients

Asthmatic patients were recruited from the Royal Brompton Hospital and University Hospitals of Leicester. Asthma severity was defined based on the Global Initiative for Asthma criteria.^{E1} Baseline characteristics of the patients with nonsevere and severe asthma are summarized in [Tables E1](#) (cohort 1) and [E2](#) (cohort 2). All patients and healthy volunteers were nonsmokers. A 50-mL blood sample from each patient or volunteer was collected into anticoagulant-containing syringes. In cohort 1 total serum IgE levels (in international units per milliliter) and blood eosinophil values (in percentages) were also determined in patients with severe asthma. This study was approved by the ethics committee of the Royal Brompton and Harefield Hospitals, the National Health Service Trust, and the Leicestershire and Rutland Ethics committee, and all subjects provided written informed consent.

Cells

The human T-cell line HUT78 and human epithelial kidney cell line HEK293 were obtained from the American Type Culture Collection (Manassas, Va) and cultured according to their guidelines. Media for HEK293 cell culture were supplemented with 200 μ g/mL G418 when cells were stably transfected. PBMCs were isolated from peripheral blood by means of density gradient separation with Ficoll-Paque Plus (GE Healthcare, Chalfont St Giles, United Kingdom) and cultured in RPMI-1640 containing 10% FBS and 15 mmol/L L-glutamine. CD4⁺ cells were separated from PBMCs by using a CD4⁺ T-cell enrichment column (R&D systems, Minneapolis, Minn). Cord blood isolated CD4⁺CD45RA⁺ T cells were obtained from STEMCELL Technologies (Grenoble, France) and cultured in RPMI-1640 containing 10% FBS and 15 mmol/L L-glutamine.

Cell lysis, immunoprecipitation, and Western blotting

Whole and nuclear cell protein extracts were prepared by using methods described previously.^{E2} Immunoprecipitation was conducted with an anti-GATA-3 antibody (Santa Cruz Biotechnology, Dallas, Tex). Cell lysates or immunoprecipitates were analyzed by using SDS-PAGE (Invitrogen, Paisley, United Kingdom) and detected by using Western blot analysis based on chemiluminescence (ECL Plus, GE Healthcare).

qRT-PCR

Total RNA was extracted with the RNeasy kit (Qiagen, West Sussex, United Kingdom), and cDNA was prepared with the M-MLV RT kit (Invitrogen, Carlsbad, Calif). Gene transcript levels of human SIRT1, IL-4, IL-5, IL-13, IFN- γ , GATA-3, T-bet, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and GNB2L1 were quantified by using qRT-PCR with commercially available TaqMan primers and probes (Applied Biosystems, Warrington, United Kingdom) on a Rotor-Gene 3000 (Qiagen).

Flow cytometric analysis

All flow cytometric analyses were performed on an Accuri C6 Flow Cytometer (BD, Oxford, United Kingdom). After treatment, CD4⁺ cells were processed for intracellular staining with the Cytofix/Cytoperm Kit (BD), according to the manufacturer's instructions, and stained with fluorescent antibodies specific to IL-4 and IFN- γ . Cells were analyzed for cytokine expression based on forward and side scatter. Quadrant markers were set based on background staining of matched isotype control antibodies and on a nonstimulated control sample treated in parallel.

SIRT1 activity

SIRT1 activity was measured with the Fluor-de-lys SIRT1 fluorometric drug discovery assay kit (Enzo Life Sciences, Exeter, United Kingdom).

Briefly, total and nonspecific activity in the presence of a high concentration of nicotinamide (1 mmol/L) were determined. Actual SIRT1 activity was calculated as total activity minus nonspecific activity, and the activity was shown as micromolar standard equivalent per microgram of protein (in units).

GATA-3 activity

GATA-3 activity was measured with the TransAM GATA Transcription factor ELISA kit (Active Motif, Rixensart, Belgium). Results were normalized against total GATA-3 expression, as determined by using Western blotting.

Acetylated GATA-3 ELISA

GATA-3 acetylation levels were determined by using a sandwich ELISA developed in house. Protein was captured with an immobilized GATA-3 antibody (Santa Cruz Biotechnology). Acetylation of GATA-3 was observed with an anti-acetylated lysine antibody (New England Biolabs, Hitchin, United Kingdom). Results were normalized against total GATA-3 expression, as determined by using Western blotting.

Overexpression of GATA-3

Plasmids containing the human GATA-3 gene were prepared with the pcDNA3.1 TOPO directional cloning system (Invitrogen). HEK293 cells were transfected with plasmid by using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Successful transformants were isolated and cultured.

T-cell stimulation

PBMCs and CD4⁺ T cells were pretreated with increasing concentrations of vehicle, sirtinol, or cambinol for 20 minutes. Cells were transferred to medium containing immobilized anti-CD3 (5 μ g/mL) and anti-CD28 (0.5 μ g/mL) and incubated at 37°C and 5% CO₂ for 24 hours. Subsequently, cells were transferred to medium containing PMA (10 ng/mL) and ionomycin (0.5 μ mol/L) for 4 hours. If cells were destined for analysis by using flow cytometry, BD GolgiPlug (BD) was added 2 hours before collection.

T_H cell differentiation

Cord blood CD4⁺CD45RA⁺ T cells (STEMCELL Technologies, Manchester, United Kingdom) were differentiated into T_H1 and T_H2 subsets according to a protocol described by Cousins et al.^{E3} Briefly, cell suspensions were transferred to 6-well plates containing a combination of immobilized anti-CD3 (1 μ g/mL) and anti-CD28 (2 μ g/mL). Human recombinant IL-12 (2.5 ng/mL) and IL-2 (10 ng/mL) and an antibody specific for IL-4 (5 μ g/mL) were added to the media to generate T_H1 cells. Human recombinant IL-2 (10 ng/mL) and IL-4 (12.5 ng/mL) and antibodies specific for IL-10 (5 μ g/mL) and IFN- γ (5 μ g/mL) were added to the media to generate T_H2 cells. After 4 days of incubation, cells were washed and resuspended in fresh media containing cytokines and antibody combinations for T_H1 and T_H2 differentiation, a process that was repeated every 7 days. When required, cells were washed and resuspended in fresh media containing PMA (5 ng/mL) and ionomycin (500 ng/mL) for 4 hours.

Dot blot analysis

PBMCs from healthy subjects were pretreated with vehicle or sirtinol (10 μ mol/L) for 20 minutes before being transferred to media containing immobilized anti-CD3 (5 μ g/mL) and anti-CD28 (0.5 μ g/mL) for 7 days. PBMCs were then transferred to fresh media for 48 hours, after which they were stimulated with PMA (10 ng/mL) and ionomycin (0.5 μ mol/L). After 24 hours, cell-culture supernatants were removed, and the Human cytokine

array Panel A (R&D Systems) was used to quantify the expression of 36 cytokines, according to the manufacturer's instructions.

Reagents

Antibodies used for detection of protein in Western blots and ELISA were anti-SIRT1 (H-300, Santa Cruz), anti-GATA-3 (D-16, Santa Cruz), anti-acetylated lysine (9441, New England Biolabs), and anti- β -actin (Abcam, Cambridge, United Kingdom). The antibody used for immunoprecipitation was anti-GATA-3 (HG3-31, Santa Cruz). Antibodies specific for IL-4 (BD), IFN- γ (Invitrogen), IL-10 (Invitrogen), CD3 (BD), and CD28 (BD) were obtained to differentiate T_H cells. For flow cytometric analysis, fluorescent antibodies specific to IL-4 (phycoerythrin; 500808), IFN- γ (allophycocyanin; 502511), and matched isotype controls were purchased from BioLegend (San Diego, Calif). PMA, ionomycin, cambinol, sirtinol, and G418 were obtained from Sigma-Aldrich (Dorset, United Kingdom); recombinant human IL-4, IL-12, and IL-2 were supplied by R&D Systems. SRT2172 was provided by Sirtris, a GlaxoSmithKline company (Cambridge, Mass). RPMI-1640, Dulbecco modified Eagle medium, FBS, L-glutamine, and sodium pyruvate were obtained from Invitrogen.

Statistical analysis

Results were expressed as means $\pm$ SEMs. Statistical analysis between the healthy and nonsevere and severe asthma groups was conducted by using ANOVA with Kruskal-Wallis analysis, and when significant, comparisons were made by using the Dunn multiple comparison test with GraphPad Prism software (GraphPad Software, San Diego, Calif). Correlation coefficients were calculated by using the Spearman rank method. One-way ANOVA, the Friedman test, and the Student *t* test were also used, when applicable.

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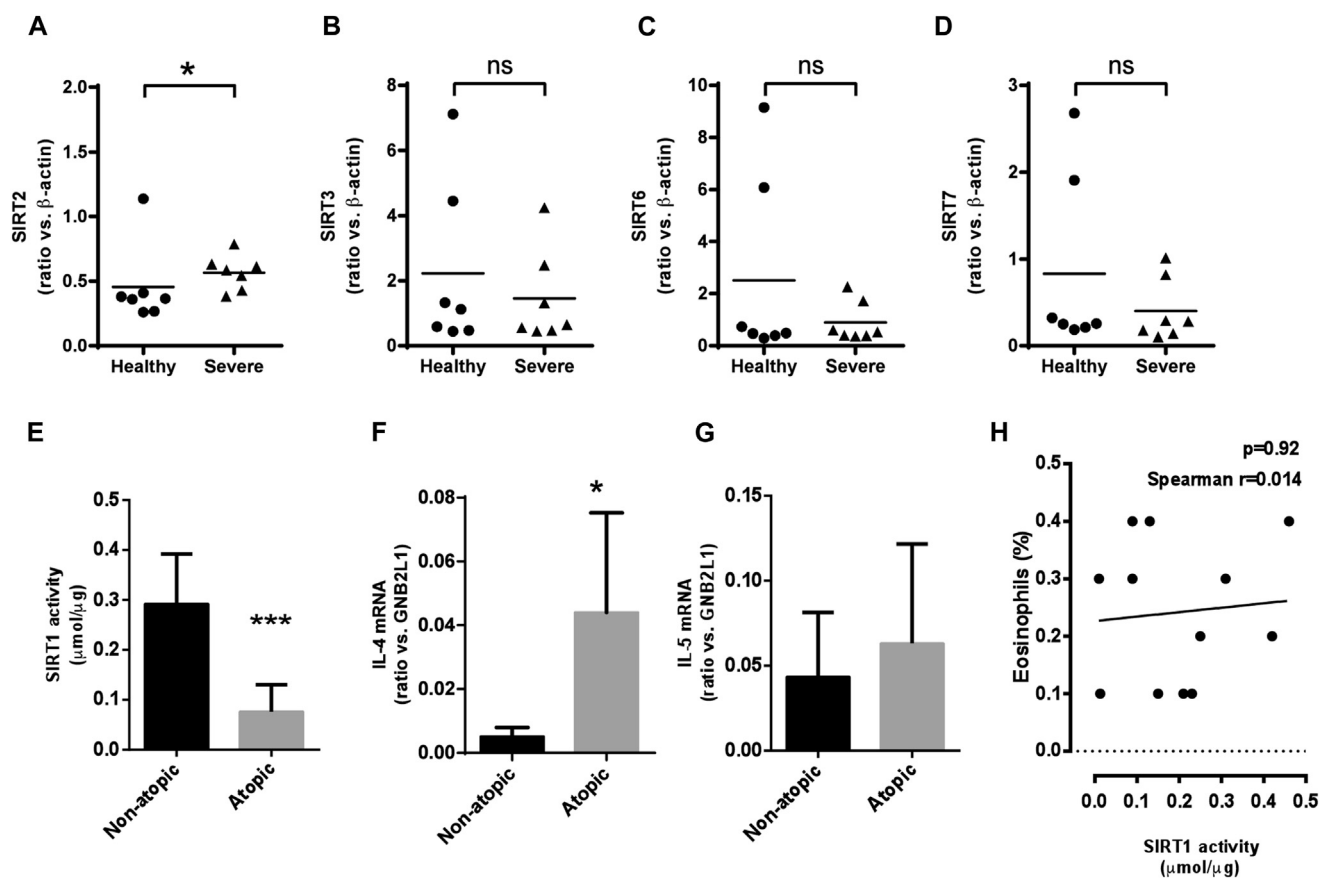


FIG E1. Sirtuin protein expression in patients with severe asthma. Protein expression was quantified by means of Western blotting (7 healthy subjects and 8 patients with severe asthma). **A**, SIRT2. **B**, SIRT3. **C**, SIRT6. **D**, SIRT7. **E-G**, Comparisons of SIRT1 activity (Fig E1, **E**), IL-4 mRNA (Fig E1, **F**), and IL-5 mRNA (Fig E1, **G**) from PBMCs between atopic and nonatopic patients with severe asthma. **H**, Spearman rank correlation analysis of SIRT1 activity and blood eosinophils (in percentages) in patients with severe asthma. * $P < .05$ and *** $P < .001$. ns, Not significant.

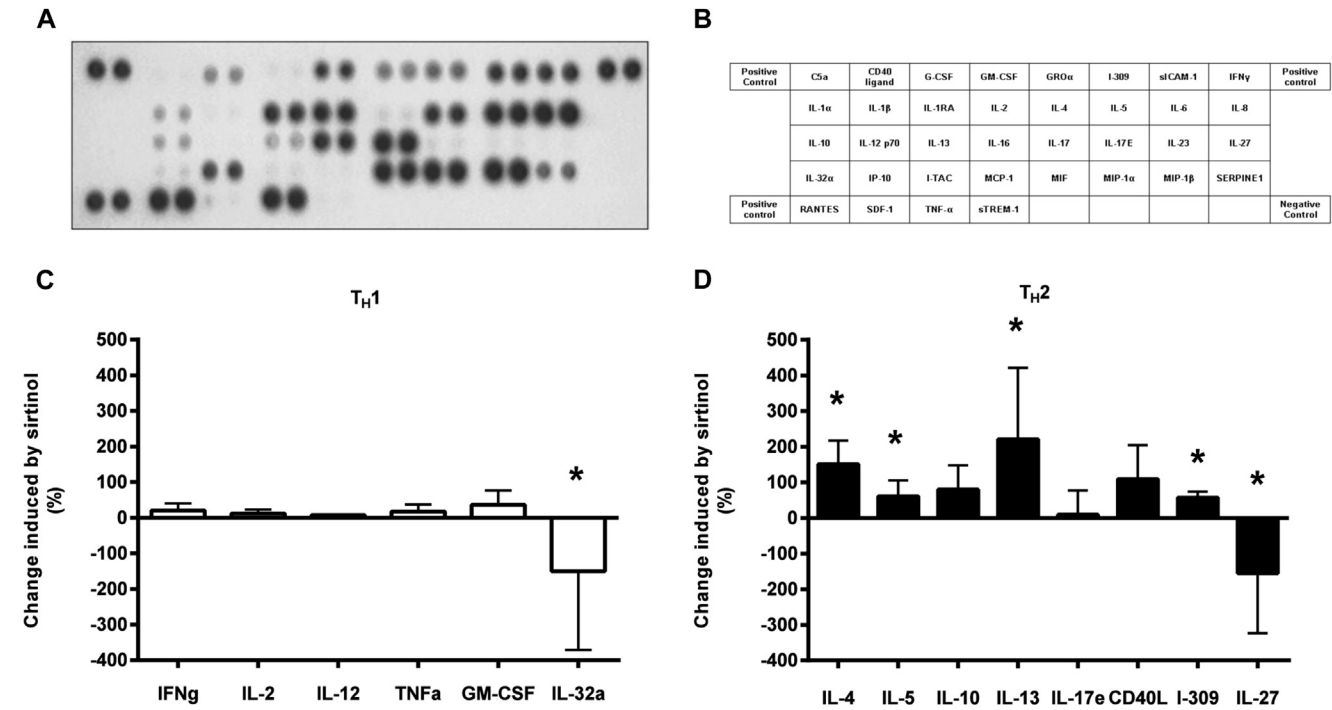


FIG E2. Effect of sirtuin inhibition on cytokine and chemokine production. **A**, Dot blot membrane. **B**, Protein layout of dot blot membrane. **C**, Expression of T_H1-associated proteins. **D**, Expression of T_H2-associated proteins. Error bars represent SEMs (n = 3). **P* < .05.

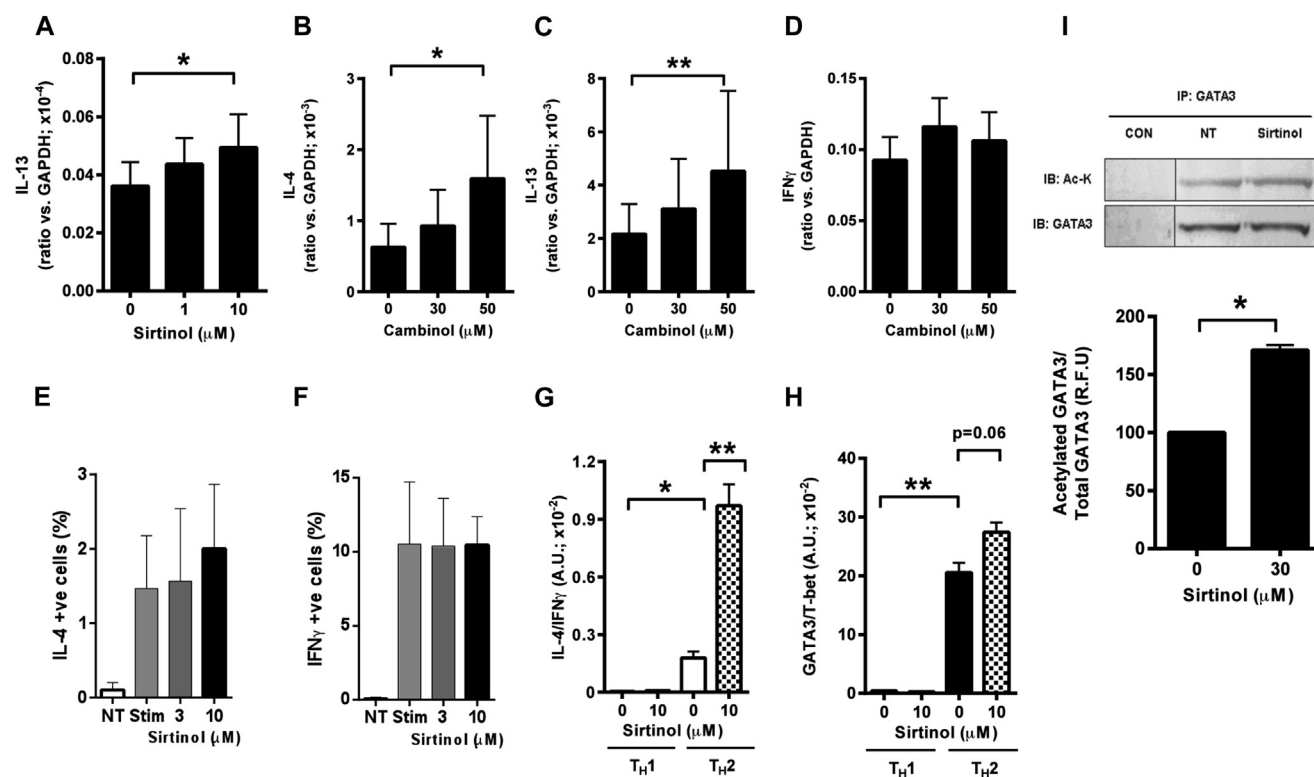


FIG E3. Inhibition of sirtuins promotes a T $_{H2}$ phenotype and increases acetylation of GATA-3. **A**, Expression of IL-13 in response to sirtuin inhibition in PBMCs ($n = 3$). **B-D**, Expression of IL-4, IL-13, and IFN- γ in response to sirtuin inhibition by cambinol in PBMCs ($n = 5$). **E** and **F**, IL-4 and IFN- γ intracellular expression in response to sirtuin inhibition by sirtinol in CD4 $^{+}$ T cells ($n = 3$). **G** and **H**, Ratio of IL-4/IFN- γ and GATA-3/T-bet in differentiated cord blood CD4 $^{+}$ CD45RA $^{+}$ T cells ($n = 4$). **I**, Acetylation of GATA-3 immunoprecipitated from HUT78 nuclear protein extract ($n = 3$). NT, Nontreatment. * $P < .05$ and ** $P < .01$.

TABLE E1. Characterization of subjects (all subjects were non-smokers) in cohort 1

	Healthy volunteers	Patients with mild asthma	Patients with severe asthma
No. (male/female)	8 (6/2)	6 (3/3)	16 (5/11)
Age (y)	35.7 ± 1.7	41.5 ± 3.3	35.1 ± 2.6
FEV ₁ (% predicted)	96.4 ± 2.2	84.5 ± 4.6*	55.0 ± 3.4†‡
Atopy	1/10	6/6	7/16
OCS	NA	0/6	11/16
ICS	NA	0/6	14/16

ICS, Inhaled corticosteroids; NA, not applicable; OCS, oral corticosteroids.

* $P < .05$ compared with healthy volunteers.

† $P < .001$ compared with patients with mild asthma.

‡ $P < .0001$ compared with healthy volunteers.

TABLE E2. Characterization of subjects (all subjects were non-smokers) in cohort 2

	Healthy volunteers	Patients with severe asthma
No. (male/female)	7 (1/6)	7 (3/4)
Age (y)	54.6 ± 2.6	45.7 ± 5.3
FEV ₁ (% predicted)	90.7 ± 3.45	76 ± 5.64*
FEV ₁ /FVC	0.80 ± 0.02	0.68 ± 0.03*
Atopy	1/8	7/7
OCS	NA	6/7
ICS		
FP	NA	6/7
BDP	NA	1/7

BDP, Beclomethasone dipropionate; *FP*, fluticasone propionate; *FVC*, forced vital capacity; *ICS*, inhaled corticosteroids; *NA*, not applicable; *OCS*, oral corticosteroids.

**P* < .05 compared with healthy volunteers.