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Targeting histone-acetyltransferase Tip60 inhibits intestinal allergy

Running title: Tip60 facilitates IgE expression

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Abstract

Background: The over production of IgE plays a critical role in the pathogenesis of allergy; the mechanism is unclear. Histone acetyltransferase (HAT) activities are required in gene transcription of a large number of molecules in the immune system of the body.

Objectives: This study tests a hypothesis that HAT Tat-interactive protein 60 (Tip60) plays an important role in the initiation of IgE-mediated allergy.

Methods: The effects of Tip60 on regulating IgE expression were assessed with B cells. An intestinal allergy mouse model was developed to assess the role of Tip60 in the induction of IgE-mediated allergic inflammation.

Results: High levels of Tip60 were observed in the peripheral B cells of patients with FA. Tip60 was required in the expression of IgE and IgG1 in B cells by inducing the chromatin remodeling at the gene locus, in which histone acetylation, signal transducer and activator of transcription 6 (STAT6) and nuclear factor- κ B at the locus of *I ϵ* promoter were markedly increased. Blocking Tip60 significantly attenuated the allergic inflammation in the mouse intestinal mucosa.

Conclusions: Tip60 plays an important role in the induction of IgE in B cells. Blocking Tip60 inhibits the allergic inflammation in the intestine, suggesting Tip60 inhibitor may be a potential anti-allergy drug.

Keywords: Allergy; Inflammation; Intestine; Tip60; B lymphocyte; IgE.

Introduction

Allergies are caused by hypersensitivity of the immune system to allergens. The immunoglobulin E (IgE) is a key mediator of allergy; it forms a complex with the high affinity IgE receptor on the surface of mast cells to make mast cell sensitized. The re-exposure to specific allergens triggers the sensitized mast cells to release allergic mediators, such as histamine, to initiate the allergy attacks (1). The IgE plays an essential role in the type I hypersensitivity, such as allergic rhinitis, allergic asthma, food allergy (FA) and allergic dermatitis (2). Although the production of IgE by B cells has been known for decades (3), the pathways by which IgE is produced and regulated are poorly understood. Our knowledge about the initiation of the IgE isotype switching at the gene locus is still limited. The therapeutic efficacy of IgE-mediated disease is to be further improved (4).

The expression of IgE by B cells is initiated by replacing the constant region of IgM by the constant chain of IgE, or the ϵ chain; this process is called IgE class, or isotype, switching (3, 5). In addition to activating the B cell receptor, other signals are also required in the IgE isotype switching; such as the transcription factors, the signal transducer and activator of transcription 6 (STAT6), nuclear factor- κ B, and signals from CD40 receptor ligation or the activation of interleukin (IL)-4 receptor (3, 5). Yet, how these signals at the IgE gene locus are organized and regulated is to be further investigated.

Histone acetyltransferases (HAT) are enzymes that acetylate conserved lysine amino acids in histone proteins by transferring an acetyl group from acetyl CoA to either the α -amino group of N-terminal amino acids or the ϵ -amino group of internal lysine residues. DNA is wrapped around by histones; the acetylation of histone elicits gene transcription (6). HAT is also

associated with immune deregulation (7). Tat-interactive protein 60 (Tip60) is one of the subtypes of HAT. It is reported that antigen stimulation leads to the increase in expression of Tip60 (8). In the allergy environment, the gene transcription is enhanced in a number of molecules, such as Th2 cytokines, IgE and IgG1. Whether Tip60 is involved in the regulation of these allergy-relevant molecule expression remains to be further investigated. Based on the above information, we hypothesize that regulation of HAT may be important in the regulation of the IgE isotype switching in B cells. Thus, we screened the expression of 13 subtypes of HAT in peripheral B cells from patients with allergic disorders and found that Tip60 was overexpressed in the B cells. Further results showed that Tip60 was necessary for the IgE isotype switching; blocking Tip60 significantly attenuated the IgE-mediated inflammation in the intestinal mucosa, suggesting a potential role of modulating Tip60 in the treatment of allergic diseases.

Materials and methods

Human subjects

Twenty FA patient and 20 healthy subjects were recruited into the present study between January 2014 and October 2016. The diagnosis of FA was carried out by physicians at the Longgang Central Hospital (Shenzhen, China) based on the established criteria at the hospital and the published guidance (9). The enrolling criteria include: patients with the FA history for at least two years. The sensitization was confirmed on the basis of a positive skin-prick test response to allergen extracts (Greer Company; Taipei, China) and the presence of serum specific IgE (≥ 0.7 kU/L; by UniCAP[®], Phadia, Sweden) (Table 1; Table S2 in supplemental materials). Patients had one of the following conditions were excluded: Cancer; using immune suppressors; undergoing immunotherapy; had severe organ diseases. The

using human tissue in the present study was approved by the Human Ethics Committee at Shenzhen University. A written, informed consent was obtained from each subject.

Preparation of naive B cells

The peripheral blood samples were collected from human subjects via ulnar vein puncture. Peripheral blood mononuclear cells (PBMC) were isolated from the blood samples by gradient density centrifugation. Single cell suspensions from the mouse spleen and gut were prepared with our established procedures (10). The immune cells were further isolated from the PBMCs or spleen cells by magnetic cell sorting (MACS) with commercial reagent kits from Miltenyi Biotec following the manufacturer's instructions. B cells were negatively selected by using biotin-conjugated monoclonal antibodies to CD11b, CD43, Thy1.2, CD138, IgG1, IgG2a/b, IgG3, IgA, and IgE along with streptavidin magnetic beads. The cells were assessed by flow cytometry. The results revealed that >98% of magnetically sorted cells were B220⁺ and CD3⁻. For plasma cell analysis, PBMCs were isolated with a plasma cell isolation kit (CD27⁺ CD138⁺) following the manufacturer's instruction.

Statistics

The data are presented as mean $\pm$ SD. The difference between two groups was determined by the Student *t* test or 2 way ANOVA along with the Bonferroni correction if more than two groups. A two-sided *p* value <0.05 was set as a statistically significant criterion.

Some experimental procedures are presented in the supplemental materials.

Results

IgE-producing plasma cells from patients with allergic disorders show high levels of Tip60

To elucidate the role of HAT in the regulation of IgE expression in plasma cells, we isolated peripheral blood mononuclear cells (PBMC) from FA patients (n=20) and healthy volunteers (n=20). The PBMCs were analyzed by flow cytometry. The results showed more plasma cells in PBMCs of the FA group than that of the healthy group (Fig. 1A-B). The frequency of IgE⁺ plasma cells was much less in the healthy group than that in the FA group (Fig. 1C). The plasma cells were then isolated from PBMCs and analyzed by RT-qPCR. The results showed that, among the 13 HAT subtypes, the Tip60 mRNA was uniquely higher in the FA group than that in the healthy group (Fig. 1D); the results were supported by the data of flow cytometry (Fig. 1E). The results imply that the Tip60 may regulate the expression of IgE in B cells.

Tip60 is required in the induction of IgE and IgG1 in B cells

To assess the role of Tip60 in the expression of IgE, we developed an *in vitro* model of IgE production following published procedures (11). After exposure to LPS/IL-4 in the culture, the mouse spleen B cells expressed IgE, IgG1 and Tip60 (Fig. 2A-E); the frequency of IgE⁺ Tip60⁺ B cell and the IgG1⁺ Tip60⁺ B cell was markedly increased (Fig. 2F-I). Next, we knocked down the Tip60 gene in B cells by RNAi (Fig. 2J); the cells were exposed to LPS/IL-4 in the culture. The enhancement of IgE and IgG1 expression was abolished in the Tip60-deficient B cells (Fig. 2A-C). To corroborate the results, an *I ϵ* promoter luciferase reporter was constructed (Fig. 2K). The constructs were transfected into B cells. The B cells were then stimulated by LPS or/and IL-4 in the culture for 48 h. High luciferase activity was detected in the B cells stimulated with both LPS and IL-4, but not to either one alone. Knockdown of

Tip60 abolished the LPS/IL-4-induced IgE promoter transactivation in B cells (Fig. 2L). The data demonstrate that Tip60 is involved in the expression of IgE in B cells.

Tip60 induces chromatin remodeling at the IgE germline promoter locus

To assess the physical interaction between Tip60 and the *I ϵ* promoter in B cells, naïve B cells were exposed to LPS and IL-4 in the culture for 3 h and analyzed by ChIP. The results showed that 30 min after the exposure of LPS, the levels of Tip60 at the STAT6 binding site and the NF- κ B binding site of the *I ϵ* promoter locus were markedly increased, which reached the top value at 60 min and was kept at a high level until 120 min, and then started to decline. The presence of an inhibitor of Tip60 abolished the increases in Tip60 at the *I ϵ* promoter locus (Fig. 3A). Since histone H3 and H4 are involved in the IgE promoter activation (12, 13), we assessed the levels of acetylated H3 (acH3) and H4 at the *I ϵ* promoter locus in the B cells. The results showed that the levels of acH3 and acH4 were increased at the STAT6 binding site and NF- κ B binding site at the *I ϵ* promoter locus 90-120 min after stimulating by LPS/IL-4, which was abolished by knocking down the Tip60 gene (Fig. 3B-E). RNA polymerase II (Pol II) catalyzes the transcription of DNA to synthesize precursors of mRNA. The exposure to either LPS or IL-4 markedly increased the Pol II at the *I ϵ* promoter locus (Fig. 3F). Furthermore, we also detected the increases in the levels of pSTAT6 at the STAT6 binding site (Fig. 3G) and the increases in the levels of pNF- κ B (p65) at the NF- κ B binding site (Fig. 3H) of the *I ϵ* promoter locus.

Modulation of Tip60 *in vivo* influences IgE expression in B cells

We next observed the expression of Tip60 in the B cells of mice. After receiving LPS intraperitoneal injection (ip), the Tip60 expression was increased in the B cells of the spleen and gut in a LPS dose-dependent manner, which was abolished by administration with an inhibitor (NU9056) of Tip60 (Fig. 4A-B). In separate experiments, mice were i.p. injected with LPS or/and IL-4. Enhancement of IgE and IgG1 expression in spleen and gut B cells was observed in mice injected with both LPS and IL-4, but not in either one alone. The increases in IgE and IgG1 in B cells induced by LPS/IL-4 could be blocked by the administration with the Tip60 inhibitor (Fig. 4C-D). In addition, we also detected that the levels of IgE and IgG1 in the sera of mice treated with LPS/IL-4 in an exposing time-dependent manner (Fig. 4E-F). The data further confirm that Tip60 plays a critical role in the IgE expression in B cells.

Inhibition of Tip60 suppresses intestinal allergic inflammation

Data reported above suggest that the overexpression of Tip60 in B cells plays a critical role in the production of IgE. IgE is the main mediator in the initiation of allergic responses. Blocking IgE can alleviate allergic inflammation (14). Thus, we next intended to inhibit intestinal allergic responses by blocking the Tip60 expression. Using OVA as an allergen, LPS as an adjuvant (15), we developed a mouse model of intestinal allergic inflammation. The mice showed high levels of serum OVA-specific IgE (Fig. 5A), serum Th2 cytokines (Fig. 5B), infiltration of mast cells and eosinophils in the intestinal mucosa (Fig. 5C), increases in OVA-specific CD4⁺ T cell proliferation in the culture (Fig. 5D), decreases in the core temperature (Fig. 5E) and had diarrhea (Fig. 5F), which were significantly attenuated by co-administration with NU9056.

Discussion

This study has revealed a previously unknown event in the regulation of IgE expression in B cells. We found that Tip60 was overexpressed in the IgE-producing B cells from patients with allergic disorders at the remission period; Tip60 was required in the IgE gene transcription and IgE production in B cells; blocking Tip60 significantly attenuated the IgE-mediated allergic inflammation in the gut mucosa.

We observed that, in the 13 subtypes of HAT, Tip60 was uniquely higher in the IgE-producing plasma cells in patients with allergic disorders. HATs function in a broad range of biological processes, such as gene regulation, dosage compensation, DNA damage repair and tumourigenesis (16). Our data reveal a novel aspect that Tip60 is involved in the regulation of IgE expression in B cells. Published data also indicate that Tip60 expression is associated with the development of IgE-mediated allergic inflammation (8). The role of Tip60 in the induction of IgE in B cells was demonstrated in this model. First, the induction of IgE in B cells by LPS/IL-4 only occurred in wild B cells, not in those Tip60-deficient B cells. Second, with the luciferase gene reporter construct technique, only B cells with high Tip60 expression produced higher levels of IgE promoter activities (in the presence of IL-4 in the culture). Third, exposure to LPS induced Tip60 expression in B cells.

STAT6 is the transcriptional factor of IgE (17), binding of IL-4 to its receptor recruits the Janus kinases, Jak1 and Jak3, which cause the phosphorylation and nuclear translocation of STAT6 to initiate the IgE expression (11). The present data indicate that exposure to LPS/IL-4 induces STAT6 phosphorylation at the ϵ promoter locus and initiates the IgE gene transcription. Another interesting point of the results is that exposure to either LPS or IL-4

alone did not increase the levels of pSTAT6 at the *I ϵ* promoter locus, suggesting a synergistic effect of LPS/IL-4 is required on the STAT6 phosphorylation. Dong et al indicate that exposure to IL-4 alone can induce STAT6 phosphorylation (18). The difference between Dong's experiments and ours may be resulted from that we used different experimental systems. They used HeLa cells, we used B cells. Their cells have been transfected with the PTB-associated splicing factor plasmids; ours are naive B cells.

The chromatin surrounding sites of DNA double-strand breaks during the IgE isotype switching and recombination (19). This process is followed by the DNA repairing. Recent studies have implicated that HAT complexes and chromatin acetylation play a critical role in DNA repair (20). The present data show that Tip60 was markedly enhanced at the IgE gene locus, implicating that the Tip60 may be involved in this process. Szumiel et al reported that histone acetylation by the Tip60 modulates loading of repair proteins and repair of DNA double-strand breaks (21). Thus, our data suggest that Tip60 is associated with the IgE isotype switching in B cells.

Previous reports show that a Tip60 mutant is not able to enhance Foxp3 suppressive activity (22). Xiao et al indicate that the selective loss of Tip60 in Foxp3 expressing regulatory T cells can lead to significant peripheral deficits of suppressive activity that lead to catastrophic scurfy like disease (23). On the other hand, Gao et al show that inactivation of Tip60 has no dramatic effects on adipose tissue or liver, nor does it alter glucose tolerance (24). Our study has uncovered another aspect of the Tip60 regulation that shows Tip60 plays a critical role in B cells to regulate the expression of IgE. It seems that Tip60 plays various roles in different cell types, which may be an interesting topic to be further investigated.

Accepted Article

In addition to the finding that Tip60 increases IgE expression in B cells, the data also show that Tip60 is associated with an increase in the IgG1 expression in B cells. This is in line with previous studies. Proper stimuli, such as exposure to both IL-4 and anti-CD40 Ab, or IL-4 and LPS, induce the IgE class switch recombination, which induces the production of both IgE and IgG1 (25). IgG1 is also involved in the pathogenesis of allergic responses (26). Some previous studies tried to administration of anti-IgE Ab to counteract the effects of IgE to attenuate allergic disorders (27). The present data implicate that administration with both anti-IgG1 Ab and anti-IgE Ab may result in better anti-allergy efficacy than using anti-IgE Ab alone. This is an interesting spot to be further investigated. Some investigators observed that specific immunotherapy suppressed antigen-specific IgE but not IgG1 (28). Data from the present study may advance this therapeutic strategy. Administration of Tip60 inhibitors may be an option to suppress both IgE and IgG1 more efficiently.

Prompted by the present data, further questions may be asked. Such as do TLR4^{-/-} mice mediate Tip60 expression? Tip60 is alternatively spliced into two isoforms termed α and β (29). Which isoform of Tip60 is required in the regulation of IgE expression? The effects of Tip60 isoform on B cells remain un-investigated. The data suggest that inhibition of Tip60 is a potential anti-allergy therapy. It is reported that Tip60^{+/-} mice display a cancer phenotype (30), does blocking of Tip60 potentially promote tumor progression? It needs to be elucidated before it can be used as a therapeutic agent. Histone acetylation is reversible, mediated by deacetylases (HDACs). The critical role of the increases in HAT/HDAC ratio in asthmatics has been described (31). Since Tip60 is overexpressed in IgE producing B cells as shown by the present data, it may be possible that specific HDACs counteract Tip60, or

regulate the expression of Tip60 isoforms, to suppress allergic inflammation, which needs to be further investigated.

In summary, the present data show that Tip60 played a critical role in the IgE expression and was associated with the initiation of allergic reactions in the body. Administration with a Tip60 inhibitor significantly inhibited the allergic inflammation in the intestinal mucosa, suggesting that the Tip60 inhibitors may have the potential to be an anti-allergy drug.

Declare of conflict of interest: None to declare.

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Author contribution:

GY, BHC, SBY, ZQL, SQQ, LTY, RDX, XRG, MGL and LG performed the experiments, analyzed data and reviewed the manuscript. ZGL and PCY organized the study and supervised the experiments. PCY designed the project and wrote the paper.

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Figure legends

Figure 1. Plasma cells of FA patients express high levels of Tip60. The peripheral blood samples were collected from 20 FA patients and 20 healthy subjects. PBMCs were isolated from the blood samples and analyzed by flow cytometry. A, selected cell population. B, flow cytometry data show the frequency of plasma cells in PBMCs. The subpanel of isotype IgG is a gating reference. C, the frequency of IgE positive cells in the plasma cells of panel B. D, the bars indicate the mRNA levels of 13 histone acetyltransferases in plasma cells. E, the gated histograms indicate the frequency of Tip60⁺ cells in the gated IgE⁺ plasma cells of panel D. The data of bars are presented as mean $\pm$ SD. *p<0.01 (*t* test), compared with the healthy group. Each experiment was repeated 3 times.

Figure 2. Tip60 is required in the expression of IgE in B cells. A-C, naïve mouse spleen B cells (10^6 cells/ml) were prepared and cultured in the presence of LPS or/and IL-4 for 6 days. The cell extracts were prepared and analyzed by RT-qPCR and Western blotting. The bars indicate the mRNA levels of IgE (A) and IgG1 (B). C, the immune blots show the protein levels of IgE and IgG1. D-E, naïve B cells (10^6 cells/ml) were treated with LPS or/and IL-4 in the culture for 48 h. The nuclear extracts were prepared and analyzed by RT-qPCR and Western blotting. The bars (D) and immune blots (E) show the levels of Tip60 expression in B cells. F-I, naïve B cells (10^6 cells/ml) were treated with LPS or/and IL-4 for 6 days and analyzed by flow cytometry. F and H: the selected cell population. G and I, the gated dot plots indicate the frequency of IgE⁺ Tip60⁺ (G) and IgG1⁺ Tip60⁺ B cells (I). The isotype IgG staining data are the gating references. J, the immune blots show the results of Tip60 RNAi. K, a sketch of an I ϵ promoter reporter. The control reporter was used a mutated I ϵ promoter sequence. L, wild and Tip60-knockdown B cells were transfected with the I ϵ promoter luciferase reporter. The cells were stimulated with LPS or/and IL-4 in the culture for 48 h. a: Cells were treated with Tip60 RNAi; b: Cells were treated control RNAi. The bars indicate the luciferase (Luc) activity. LPS = 10 μ g/ml. IL-4 = 50 ng/ml. The data of bars are presented as mean $\pm$ SD. *, $p < 0.01$ (t test), compared to the LPS group (A, B) or saline group (D, I). The data represent 3 independent experiments.

Figure 3. Tip60 alters the chromatin at I ϵ promoter sites in B cells. B cells (10^6 cells/ml) were stimulated with LPS and IL-4 in the culture. The cells were collected at 6 different time points respectively from 0 min to 180 min and analyzed by ChIP. The bars indicate the levels of the molecules denoted on the Y axis at the STAT6 binding site and NF- κ B in I ϵ promoter locus in the B cells. Tip60 shRNA (control shRNA): B cells were treated with shRNA of Tip60

(or control shRNA) to knock down the Tip60 gene and then were treated with LPS/IL-4 for 90 or 120 min in the culture. LPS = 10 μ g/ml. IL-4 = 50 ng/ml. The “LPS” and “IL-4” in panels G and H indicate that B cells were treated with LPS alone or IL-4 alone. The data of bars are presented as mean $\pm$ SD. *, $p < 0.01$ (t test), compared with the time point 0. The data are summarized from 3 independent experiments.

Figure 4. Tip60 is involved in IL-4/LPS-induced IgE expression in B cells *in vivo*. A-B, grouped naïve BALB/c mice (6 mice per group) were intraperitoneally injected (i.p.) with LPS. The doses of LPS are denoted on the X axis of panel A. The mice were sacrificed 48 h later. B cells were isolated from the mouse spleen and the small intestine, respectively, by MACS. The B cell extracts were analyzed by RT-qPCR and Western blotting. The bars show the mRNA levels of Tip60. B, the Western blots show the protein levels of Tip60. C-D, naïve BALB/c mice were i.p. injected LPS (5 mg/kg) and IL-4 (50 μ g/kg) with or without i.p. injection with NU9056 (inh; 0.4 mg/kg). DMSO: The dissolvent of NU9056. The mice were sacrificed 48 h later. B cells were isolated from the spleen and gut; the cellular extracts were analyzed by RT-qPCR. The bars show the mRNA levels of IgE (C) and IgG1 (D). E-F, LPS/IL-4-treated mice were sacrificed at time points of 0-48 h respectively. The sera were collected and analyzed by ELISA. Each sample was analyzed in triplicate. The bars indicate the serum levels of IgE (E) and IgG1 (F). Samples from individual mice were processed separately. * $p < 0.01$ (t test), compared with the “0” group (A, E, F) or the saline group (C, D). Each experiment was repeated 3 times.

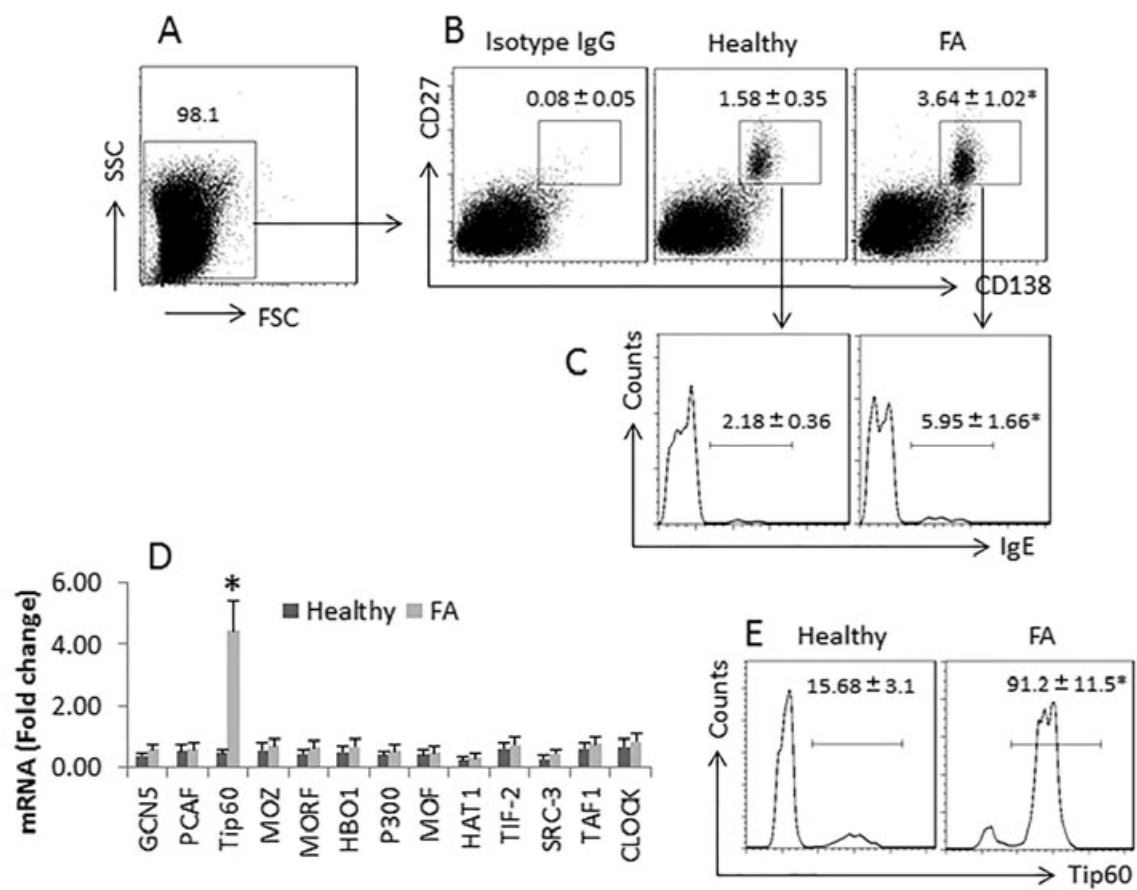
Figure 5. Blocking Tip60 inhibits gut allergic inflammation. A gut allergic inflammation mouse model was developed. A-B, the bars show the serum levels of OVA-specific IgE (A)

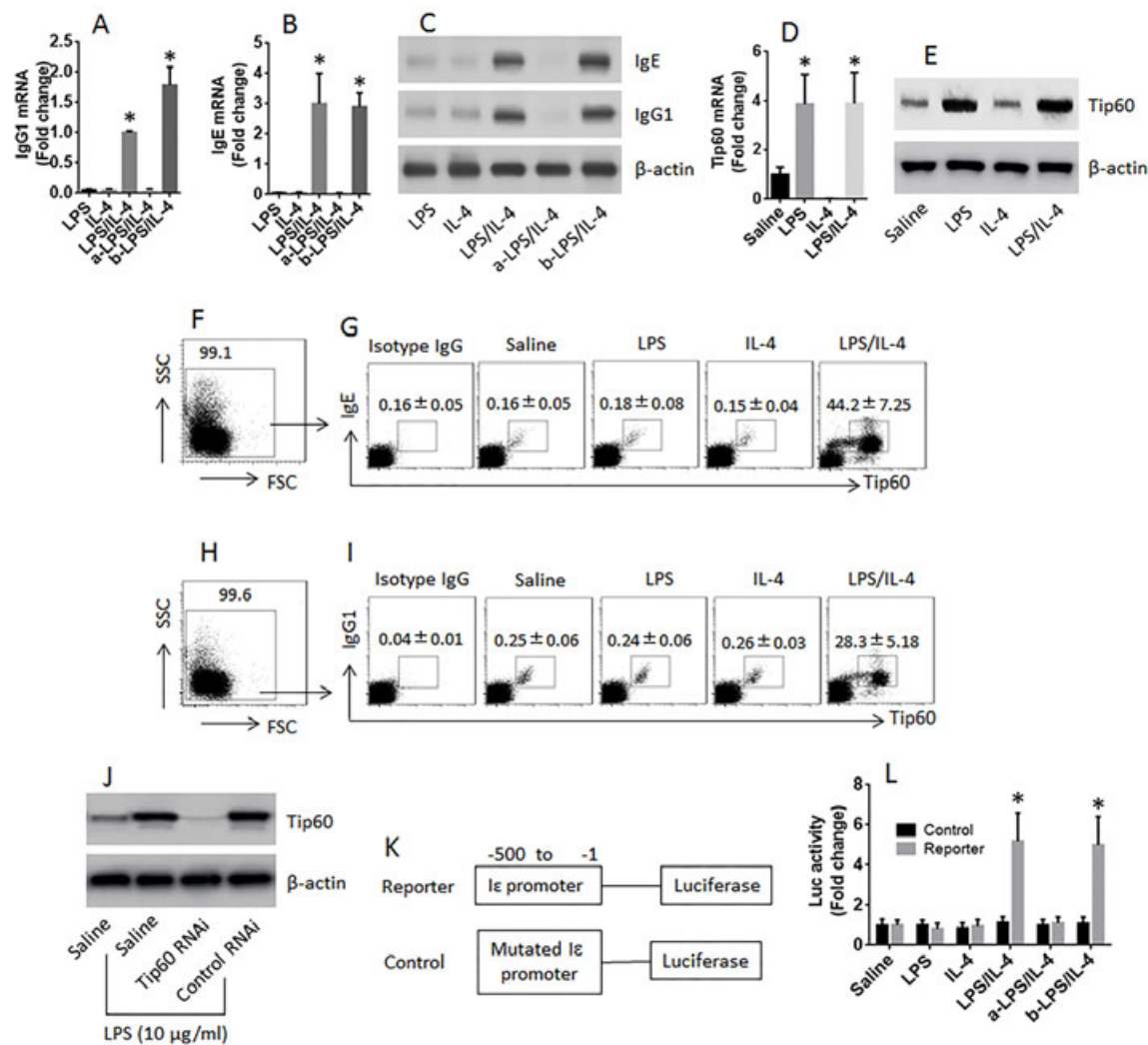
and Th2 cytokines (B). C, the bars show the frequency of mast cell and eosinophil in the gut mucosa (averaged from 20 high power fields per mouse). D, the bars indicate the summarized data of proliferating intestinal CD4⁺ T cells (the representative histograms are presented in sFig. 4 in supplemental materials). Challenge: CD4⁺ T cells were challenged with OVA or BSA in the culture. E, the bars show the changes of the core temperature (measured at 30 min after the specific allergen challenge). F, the bars show the number of mice with diarrhea. Serum samples from individual mice were processed separately. Each sample was analyzed in triplicate. NU9056: Allergic mice were i.p. injected with NU9056 (Tip60 inhibitor; 0.3 mg/kg) 30 min prior to each exposure to OVA. DMSO: The dissolvent of NU9056. The data of bars are presented as mean $\pm$ SD. *, $p < 0.01$ (t test), compared with the naïve group. Each group consists of 6 mice.

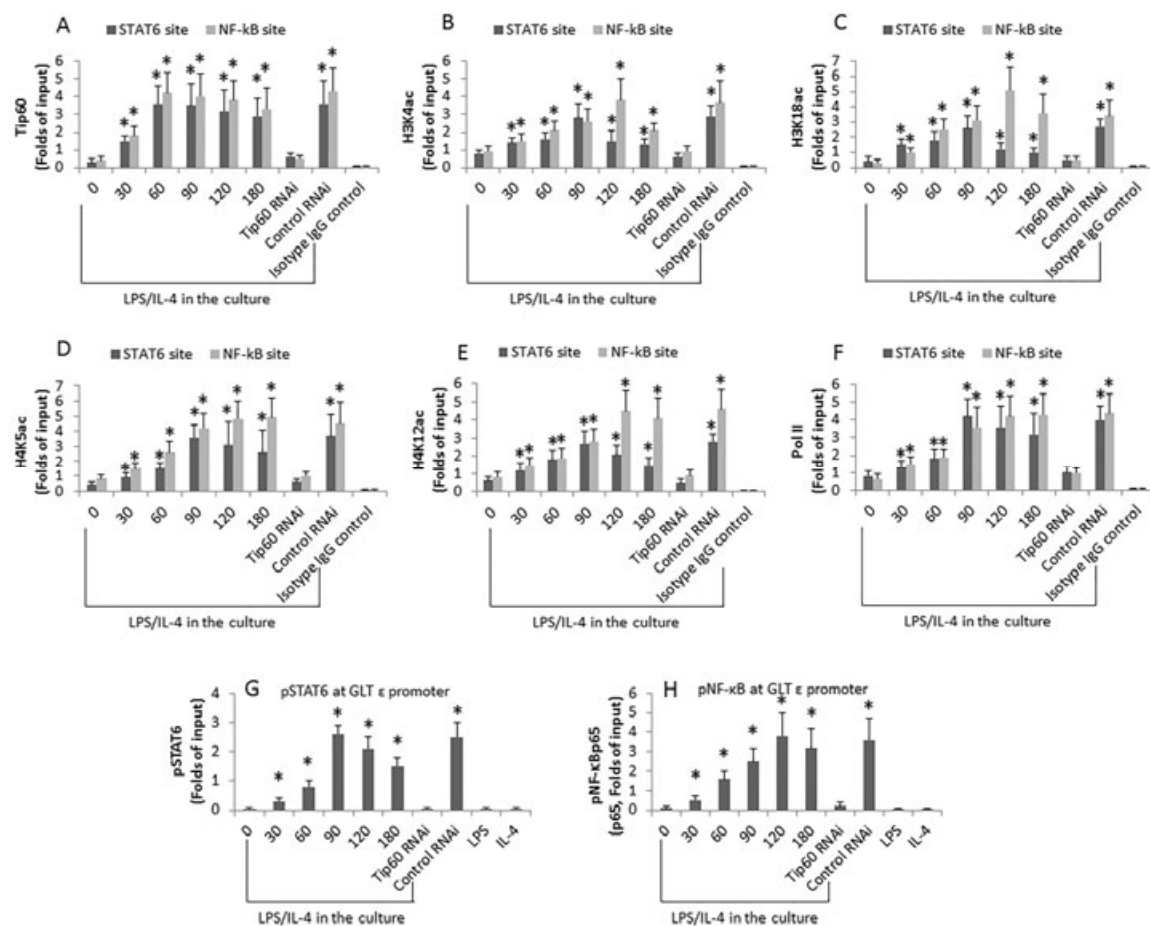
Table 1. Demographic data

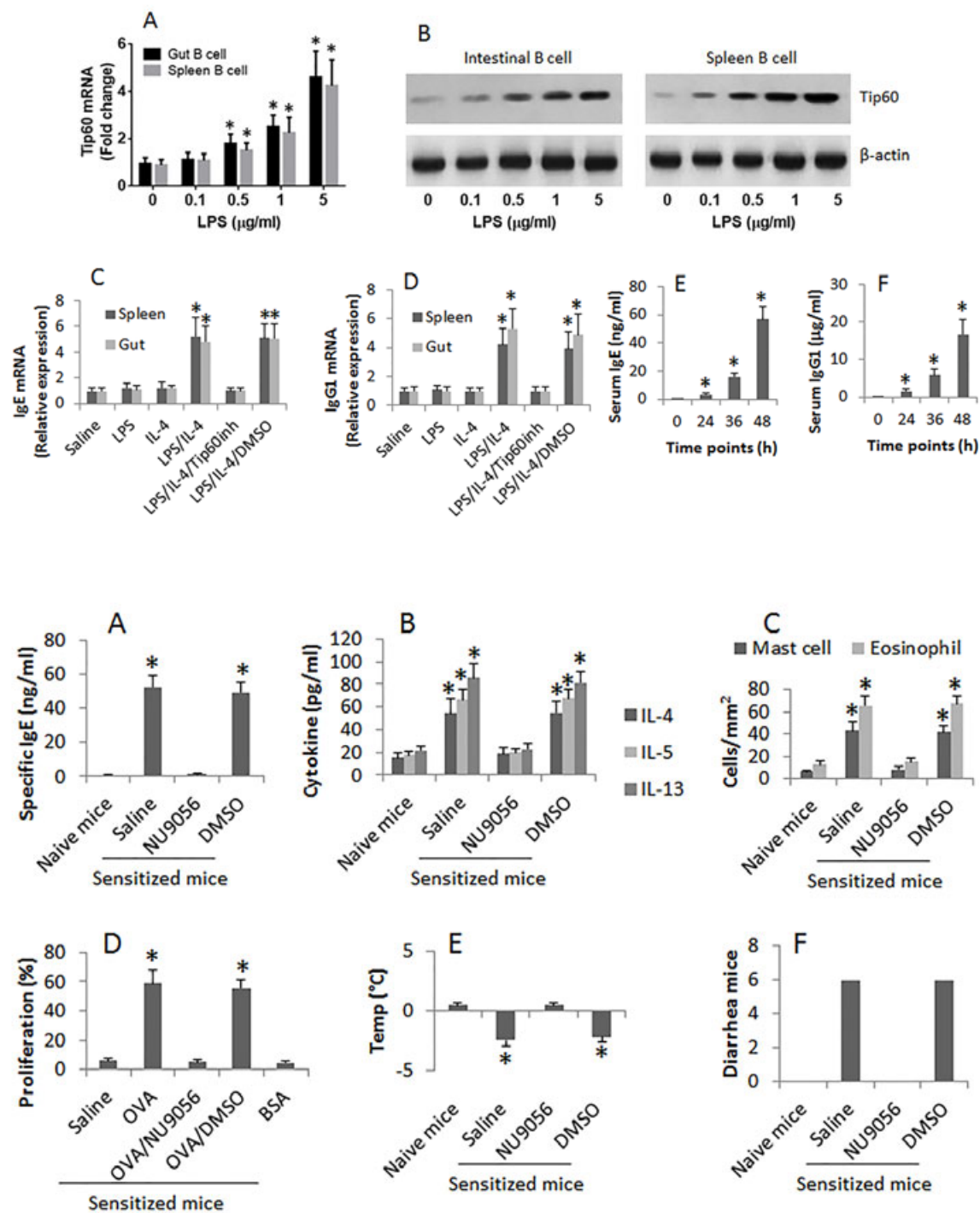
	Healthy	FA
Male	10	10
Female	10	10
Age (year)	29.7 $\pm$ 5.2	31.5 $\pm$ 6.5
Allergy history (year)	No	3.1 $\pm$ 2.8
Serum specific IgE(kU/L)	UD	7.28 $\pm$ 4.27
Serum total IgE (kU/L)	0.61 $\pm$ 0.48	9.54 $\pm$ 4.25*
Serum specific IgG1 (g/L)	0.82 $\pm$ 0.53	5.15 $\pm$ 3.22*
Serum total IgG (g/L)	7.82 $\pm$ 4.31	9.17 $\pm$ 4.08

*, $p < 0.01$, compared to the healthy group. UD: Under detectable levels.









Supplemental materials

Targeting histone-acetyltransferase Tip60 inhibits intestinal allergy

Running title: Tip60 facilitates allergy

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Reagents

The antibodies of CD40, Tip60, H3K4, H3K18, RNA polymerase II, STAT6, pSTAT6, IgG1, IgE and the Tip60 shRNA kit were purchased from Santa Cruz Biotech (Santa Cruz, CA). The fluorochrome-labeled antibodies of CD19 and CD138 were purchased from BD Biosciences (Franklin Lakes, NJ). The LPS, OVA, NU9056 (a Tip60 inhibitor; was dissolved in DMSO as 10 mM stocks and stored at -20°C) and ChIP reagent kit was purchased from Sigma Aldrich (St. Louis, MO). The ELISA kits of OVA-specific IgE, total IgE, IgG, IL-4, IL-5 and IL-13, and IL-4 proteins were purchased from Dakewe Biomart (Beijing, China). The reagents for real time RT-PCR, Western blotting and gene transduction were purchased from Invitrogen (Carlsbad, CA). The immune isolation reagent kits were purchased from Miltenyi Biotech (San Diego, CA). The permeabilization in Fix/Perm buffer was purchased from eBioscience (San Diego, CA). The endotoxin levels in all reagents were detected using the Limulus assay (Limulus amoebocyte lysate QCL 1000, Bio Whittaker, Walkersville, MD, USA). The reagents used in this study contained <0.2U endotoxin/10 µg reagents.

Flow cytometry

For the surface staining, cells were incubated with fluorochrome-labeled antibodies, or isotype IgG, for 30 min at 4 °C. If necessary, after the surface staining, the cells were treated by fixation and permeabilization in Fix/Perm buffer for 60 min. After washing with PBS, the cells were stained with fluorochrome-labeled antibodies, or isotype IgG, for 60 min at 4°C. The cells were analyzed with a flow cytometer (FACSCanto II). The data were analyzed by Flowjo software

(TreeStar, Ashland OR). 100, 000 cells per sample were analyzed. Data from isotype IgG-staining were used as a gating reference.

Real time RT-PCR (RT-qPCR)

Following published procedures (PLoS One. 2013; 8: e84944), total RNA was extracted from the B cells with TRIzol reagents (10^6 cells/sample of B cells; 10^5 cells/sample of plasma cells). The cDNA was synthesized with the SuperScript® IV first-strand synthesis system according to the manufacturer's protocol. qPCR was performed in a real time PCR device (MiniOpticon, Bio Rad) with the cDNA using the SYBR Green Master Mix with the primers listed in Table S1. All samples were analyzed in triplicate. The results were normalized to the relative mRNA expression against the housekeeping gene (β -actin) as reported (Nature Medicine. 2015; 21: 1357-63). The relative mRNA expression level of each gene was averaged from triplicate measurements of 3 independent experiments and presented as the relative expression.

Preparation of cytosolic and nuclear extracts

Cells were incubated with lysis buffer (10 mM HEPES, pH 7.4, 10 mM NaCl, 1.5 mM MgCl₂, 0.5 mM DTT, 0.2% Nonidet P-40, and 0.2 mM PMSF) at 4°C for 15 min, and centrifuged at 500 ×g for 10 min at 4°C. The supernatant was collected as the cytosolic extract. The pellet was added with nuclear extract buffer (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, and 1× protease inhibitor cocktail) and incubated for 15 min at 4°C, followed by centrifugation at 13,000 ×g for 10 min at 4°C. The

supernatant was collected as the nuclear extract. The protein concentrations were determined by the Bradford method.

Western blotting

Protein (50 µg/well) was separated on the SDS-polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane. The membranes were blocked with 5% skim milk for 1 h at room temperature and were subsequently incubated with the primary antibodies (300 ng/ml) or isotype IgG overnight at 4°C. The membranes were washed with Tris-buffered saline supplemented with 0.1% Tween 20 (TBST) for 3 times and followed by incubation with the peroxidase-conjugated secondary antibodies for 1 h at room temperature. The membranes were then washed with TBST three times and developed with an ECL (enhanced chemiluminescence) Western blotting detection system. The results were photographed with an image station (UVI, Shenzhen, China).

Mice

Male BALB/c mice (6-8 week old) were purchased from the Guangzhou Experimental Animal Center (Guangzhou, China) and maintained in a pathogen-free environment. The animal experimental procedures were approved by the Animal Ethic Committee at Shenzhen University. The experiments were performed in accordance with the guidelines for animal care.

Preparation of luciferase reporter constructs of the I β promoter

The AdFluc reporter of I ϵ promoter were constructed by the Sagon Biotech (Shanghai, China) with I ϵ promoter sequence from -500 to -1. A mutated I ϵ promoter sequence (“gcatcctaaa” at -194 to -183 was mutated to “gtatcaaaaa”; “aacatcctcc” at -134 to -123 was mutated to “aat~~tt~~cctac”; the replaced nucleotides are highlighted) was used to construct the control AdFluc reporter. The constructs were transduced into B cells following the manufacturer’s instructions. At 48 h post infection B cells were lysed and collected for luciferase activity assay using the Luciferase Assay Kit (Promega, Madison, WI). Each treatment/assay condition was performed in triplicate. For the reporter-gene analysis, that number of cells subjected for reporter gene analysis were adjusted to same protein level using the Bradford method (Bio-Rad, Hercules, CA).

Chromatin immunoprecipitation (ChIP)

Proteins at the I ϵ promoter locus were analyzed by ChIP assay that was performed with the ChIP kit and protocol from Sigma Aldrich. After fixing with 1% formaldehyde for 15 min, the cells were lysed and sonicated to shear the chromatin DNA into short fragments. Cell lysates were precleared with protein G-agarose beads. The supernatant was collected and incubated with the antibodies of interest or isotype IgG overnight at 4°C. The antibody-chromatin complex was precipitated by incubation with protein G-agarose beads for 1 h at 4°C. The beads were collected by centrifugation and washed. The complexes on the beads were eluted with elution buffer. DNA in the complexes was recovered by reverse crosslinking at 65°C for 4 h. The lysates (10%; before antibody precipitation) were used as the input. qPCR was carried out with the primers of the I ϵ promoter region, including STAT6 binding sequence (acctgggatcagacgatggagaat and agcagccttgggaagttcccttt) and NF- κ B binding sequence

(aagggaacttccaaggctgctaag and accattaattcatgccagatgtagt). The results were calculated and presented as folds of change against the input.

Enzyme-linked immunosorbent assay (ELISA)

The levels of Th2 cytokines, OVA-specific IgG1 and OVA-specific IgE were determined by ELISA with commercial reagent kits (Th2 cytokine kits, total IgE kits and IgG1 kits were purchased from R&D Systems. OVA-specific IgE kits were purchased from Beijing Lvyuanbode Biotech) following the manufacturer's instructions. Serum specific IgE levels were determined by UniCAP® (Phadia, Sweden).

Isolation of lamina propria mononuclear cells (LPMC)

Immediately after sacrifice, the small intestine was removed from mice. The intestine was cut longitudinally and washed three times in PBS. The intestine was cut into 2.0 cm long pieces and incubated in a solution containing 5 mM EDTA (Sigma, St. Louis, MO) and 1 mM dithiothreitol (DTT) (Sigma) in Hanks' balanced salt solution (HBSS) (GIBCO, Gaithersburg, MD) for 30 min at 37 °C. The tissue was then treated by enzymatic digestion with 1 mg/ml collagenase D (Roche Diagnostics, Indianapolis, IN) in calcium magnesium free HBSS for 45 min. After isolation, cells were passed through a 100 µm and a 40 µm filter. The cells were resuspended in 44% Percoll, then underlaid successively with 50% and 67% Percoll in a conical tube. Gradients were centrifuged at 400×g for 30 min at room temperature. Following centrifugation, the 44/50 and 50/67 interfaces were collected and combined. They were washed twice in culture medium. The CD19⁺ B220⁺ CD3⁻ CD11b⁻ B cells were isolated from the PBMCs by MACS. The CD3⁺ CD11c⁺

cells were selected out first. From the remainder the CD19⁺ B220⁺ B cells were positively selected. The cells were assessed by flow cytometry. The results revealed that the purity of B cells was >98%.

Intestinal mast cell and eosinophil staining

Immediately after sacrifice, one segment of the jejunum was cut and fixed with 4% formalin overnight. The intestinal tissue was embedded in paraffin and cut to 4 µm sections, and stained with hematoxylin and eosin (for eosinophil counts) or 0.5% toluidine blue (for mast cell counts). The sections were observed with a light microscope. The mast cells and eosinophils in the sections were counted in 20 high power (×400) fields per mouse. The results were presented as cell number per mm² tissue. The slides were coded. The observers were not aware of the code to avoid the observer bias.

Assessment of antigen-specific T cells in mice

To assess the antigen-specific CD4⁺ T cell proliferation, CD4⁺ T cells and dendritic cells were isolated by MACS from the spleen of allergic mice. The CD4⁺ T cells (1×10^6 /ml) were labeled with 0.5 µM carboxyfluorescein succinimidyl ester (CFSE, Life Technologies) and cultured with dendritic cells (T cell: DC = 5: 1) in the presence of OVA (5 µg/ml; the specific antigen) or bovine serum protein (5 µg/ml; an irrelevant antigen used as a control) for 3 days. The cells were analyzed by flow cytometry.

Cell culture

The isolated cells were cultured in RPMI1640 medium (Sigma Aldrich) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 2 mM L-glutamine. In the case of B cell culture, anti-CD40 (100 ng/ml; Santa Cruz Biotech) was added to the culture. The viability of the cells was greater than 98% before using for further experiments as checked by the Trypan blue exclusion assay.

RNA interference (RNAi)

Knockdown of Tip60 expression was performed using the short hairpin-activated gene silencing system purchased from Santa Cruz Biotech following the manufacturer's instructions. The shRNA specific for Tip60 was a combination of three sequences targeting different segments of Tip60 mRNA, allows for long-term suppression of Tip60 gene expression {Hong, 2015 #50}. At 3 days after transfection with the Tip60 shRNA or control shRNA, cells were washed and then followed by the puromycin selection (1 µg/ml). The effect of RNAi was determined by Western blotting and flow cytometry (Fig. S1).

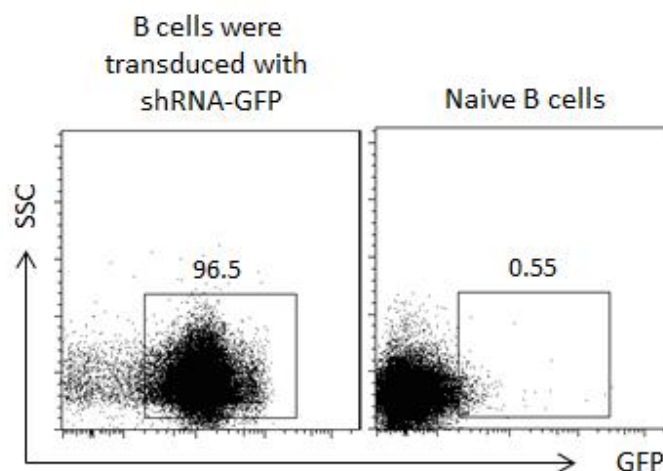


Fig. S1. Assessment of the efficiency of Tip60 shRNA transduction. Naive B cells were transduced with Tip60 shRNA-GFP. The cells were analyzed by flow cytometry 48 h later. The gated dot plots indicate the Tip60 shRNA-loaded B cells. The naive B cells were used as negative controls. The experiment was repeated 3 times.

Development of a mouse model of allergic inflammation in the gut mucosa

BALB/c mice were subcutaneously injected with ovalbumin (OVA; 4 mg/kg) mixing with LPS (10 µg/kg) in 0.1 ml alum on day 1 and day 7 respectively. The mice were orally challenged with OVA (40 mg/kg) in 0.1 ml saline on day 9, 11 and 13 respectively. The mice were sacrificed on day 14. Samples of the blood, the small intestine, the spleen were collected for further assessment. The core temperature was recorded at 30 min, and diarrhea was recorded during 2 h after the last allergen challenge. To test the effect of Tip60 in the induction of gut allergy, a group of allergic mice was treated with 1,2-bis (isothiazol-5-yl) (NU9056; an inhibitor of Tip60 disulfane) 0.3 mg/kg 30 min prior to each exposure to OVA. The assessment of the allergic inflammation in the gut mucosa was following our established procedures (Zhang et al. Sci Rep 3:3345).

Table S1. Primers used in the present study

Molecule	Forward	Reverse
GCN5	tccccattgacctgaagacc	cctccctccttgagcttgaa
PCAF	cctcttctggacttgaggca	tggtctctggtccaagcatt
Tip60	ctacaatgtggcctgcatcc	ttgatggtgatctgtgcct
MOZ	tgcccaatcctttctaagcc	acactacttactgagcgccg
MORF	cttaatggactgtggcgctcg	ctgctctcctgggtcaaaga
HBO1	ccacgaactcctcctccatt	agtcagggattgtggtgagg
P300	ttgtgaagagcccatggat	gctttgcatcactgggtcaa
SRC-1	ggtaaaaggtgcaggtgtgg	acaaagtacggggaaggagg
HAT1	gtgtaccagacaaaacccg	ccgggaaaaacagggc aaat
TIF-2	gttttgccagccacttacca	aacacactggcgttttctcc
SRC-3	acatgggagtcctggtcttg	ctggaaaagctgtggttccc
TAF-1	aagttcctgggcctaactcc	ctcttcggatgctgctttc
CLOCK	gtgttacatcgcgccatcat	cattaggagggtgagaggg
IgE (mouse)	atccagacagtgtgaagggg	tgactgaggttccttgacct
IgG1 (mouse)	gtacaccattccacctcca	cgtgttcatgatgggctgag
Tip60 (mouse)	actggctgacaagtacccaa	ttgatggtgatctgtgcct

Table S2. Results of skin prick test in allergy patients

Patient#	Wheat	Egg	Soy	Milk	Peanut	Cod
1		+				
2		+				
3	+		+			
4		+				
5			+	+		
6		+				
7	+	+	+			
8	+			+		
9		+				
10				+		
11				+	+	
12		+				
13						+
14	+	+				
15		+				
16					+	
17				+		
18		+				
19		+				
20		+				
Total (%)	4 (20)	12 (60)	3 (15)	5 (25)	2 (10)	1 (5)