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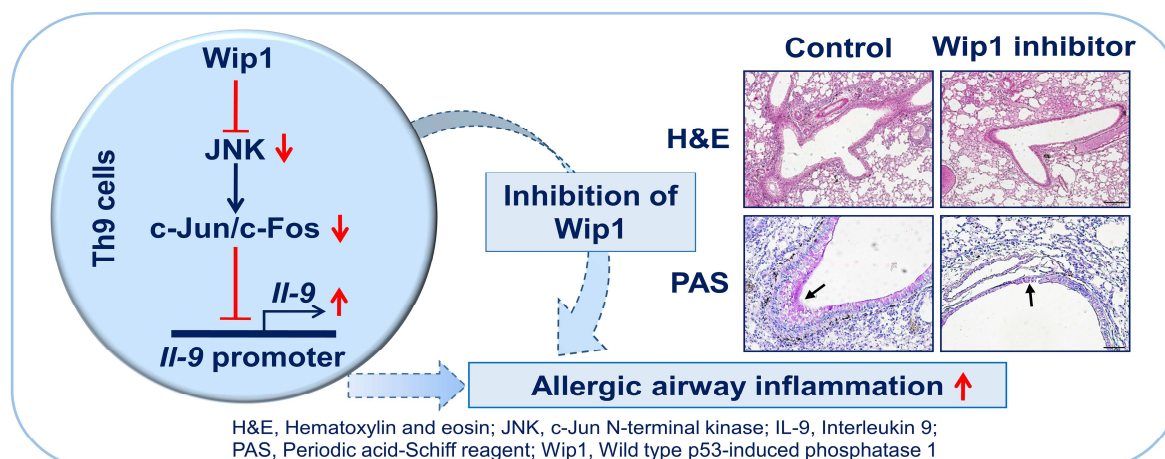
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Phosphatase Wip1 controls the development of Th9 cells and allergic airway inflammation

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Abstract

Background: Allergic asthma is one of the most common diseases worldwide, resulting in a burden of diseases. No available therapeutic regimens can cure asthma so far.

Objective: To identify new molecular targets for Th9 cells-mediated allergic airway inflammation.

Methods: Wild-type p53-induced phosphatase 1 (Wip1) gene knockout mice, Wip1 inhibitor-treated mice and ovalbumin (OVA)-induced allergic airway inflammation mouse models were employed to characterize the roles of Wip1 in allergic airway inflammation. The induction of Th cell subsets *in vitro*, real-time PCR, immunoblots, luciferase assay and ChIP assays were employed to determine the regulatory pathways of Wip1 in Th9 differentiation.

Results: We herein demonstrate that Wip1-deficient mice are less prone to allergic airway inflammation as indicated by the decreased pathological alterations in lungs. Short-term treatment of Wip1-specific inhibitor significantly ameliorates allergic inflammation progression. Intriguingly, Wip1 selectively impaired Th9 cell but not Th1, Th2 and Th17 cell differentiation. Biochemical assays show that Wip1 deficiency increases c-Jun/c-Fos activity in a JNK-dependent manner and that c-Jun/c-Fos directly binds to *IL-9* promoter and inhibits *IL-9* transcription.

Conclusion: Phosphatase Wip1 controls Th9 cell development through regulating c-Jun/c-Fos activity on *IL-9* promoter and is important for the pathogenesis of allergic airway inflammation. These findings shed lights on the previously unrecognized roles of Wip1 in Th9 cell differentiation. The inhibitory effects of a Wip1 inhibitor on the pathogenesis of allergic airway inflammation

may have important implications for clinical application of Wip1 inhibitors in allergy therapies.

Key Messages

- Phosphatase Wip1 specifically regulates Th9 cell differentiation through JNK-c-Jun/c-Fos signal pathway.
- Wip1-deficient mice showed alleviative allergic airway inflammation with less Th9 cells *in vivo*.
- Wip1 inhibitor treatment significantly prevents the pathogenesis of allergic airway inflammation in mice.

85 **Capsule Summary**

86 Phosphatase Wip1 specifically regulates Th9 cell differentiation and Wip1
87 inhibition may represent a promising strategy to treat allergic airway
88 inflammation.

89

Key words: allergy; Wip1; Th9 cell development; IL-9; phosphatase

Abbreviations:

Wip1, Wild type p53-induced phosphatase 1;

Th, T-helper;

ILC2s, type 2 innate lymphoid cells;

iTreg, induced regulatory T cells;

GATA3, GATA binding protein 3;

IRF4, interferon regulatory factor 4;

p38MAPK, p38 mitogen-activated protein kinase;

NF- κ B, nuclear factor kappa B;

AP-1, activator protein 1;

MAPK, Mitogen-activated protein kinases;

STAT, signal transducer and activator of transcription;

NFAT, nuclear factor of activated T cells;

PBS, Phosphate Buffered Saline;

FBS, Fetal bovine serum;

FACS, Fluorescence Activated Cell Sorter;

OVA, Ovalbumin;

MTA, methylthioadenosine;

PAS, Periodic acid Schiff reagent;

H&E, Hematoxylin and eosin;

BAL, bronchoalveolar lavage;

dLNs, draining lymph nodes.

Introduction

Allergic asthma is one of the most common chronic diseases, resulting in a substantial burden of diseases. Although rates of deaths due to asthma worldwide have shrunk greatly over the past 25 years, no available therapeutic regimens can yet cure this complex disease. Current clinical therapies are based on the inhalation of corticosteroids and long-acting β 2 agonists, and are effective in controlling asthma in most, but not all patients¹. Asthma is a chronic inflammatory disease of the airways characterized by the infiltration of eosinophils, basophils, mast cells, and CD4 T-helper (Th) cells into the airway submucosa, causing the inflammation of the lungs. Th2 cells and type 2 innate lymphoid cells (ILC2s), which produce IL-4, IL-5, and IL-13, are now recognized as important mediators of allergic eosinophilic asthma^{2, 3}. Other important players are innate immune cytokines such as IL-1, IL-25 and IL-33, as well as thymic stromal lymphopoietin. They are released from airway epithelial cells and can in turn activate Th2 cells and/or ILC2s^{3, 4}. Another important cytokine is IL-9, which expression is higher in lungs of asthmatic patients^{5, 6}. Moreover, transgenic expression of IL-9 has been shown to promote allergic inflammation⁷⁻⁹. IL-9 increases mucus production from lung epithelial cells and pulmonary mastocytosis¹⁰. More evidences of the importance of IL-9 in the development of allergic airway inflammation come from an acute asthma model using ovalbumin (OVA)/alum immunization followed by aerosolized airway OVA challenges as a trigger for asthma development. Using this model, Cheng et al found a significant decrease in airway hyper-responsiveness and BAL eosinophilia after administration of an IL-9 blocking antibody¹¹. Additionally, a recent study showed that Th9 cells

were involved in the lung allergic inflammation¹². Adoptively transferred Th9 cells lead to allergic airway disease characterized by increased amounts of eosinophil recruitment, tissue mast cell numbers, and serum anti-OVA IgE levels following OVA challenge¹³⁻¹⁵, supporting the contribution of Th9 cells to allergic inflammation. However, it is also reported that IL-9 may not significantly impact allergic airway inflammation in mice¹⁶. Thus, the inconsistency still needs to be clarified.

Depending on the surrounding cytokine milieu, naive CD4⁺ Th cells are activated and differentiate into distinct subsets including Th1, Th2, Th9, Th17 cells or induced regulatory CD4⁺CD25⁺ T cells (iTreg), which are characterized by differential cytokine secreting profiles^{17, 18}. Interleukin 9 (IL-9)-producing CD4⁺ helper T cells (Th9 cells) are induced from naive CD4⁺ T cells by transforming growth factor- β (TGF- β) and IL-4 from naive CD4⁺ T cells under the stimulation of the T cell antigen receptor (TCR). Th9 cells have been shown to participate in allergic inflammation, autoimmune diseases and cancers. TGF- β alone without IL-4 promotes iTreg cells by inducing Foxp3^{19, 20}, whereas IL-4 alone supports Th2 induction. IL-4 activates signal transducer and activator of transcription (STAT) 6 and interferon regulatory factor 4 (IRF4), whereas TGF- β activates PU.1 and represses T-box expressed in T cells (T-bet) and GATA binding protein 3 (GATA3). It is the integration of those events that eventually drives IL-9 expression, as either of these cytokine alone induce the differentiation into either Tregs or Th2²¹. Sequence and molecular analysis identified the potential binding sites for transcription factors including PU.1, IRF4, STATs, nuclear factor of activated T cells (NFAT), GATA1, GATA3,

Smads and Notch as well as nuclear factor kappa B (NF- κ B) and activator protein 1 (AP-1) in the promoter region of *Il-9*^{12, 21, 22}. However, it remains elusive about the molecular regulatory network of Th9 differentiation so far.

Wild-type p53-induced phosphatase 1 (Wip1, also called PP2C δ or PPM1D) is a serine/threonine protein phosphatase, which belongs to the type 2C δ protein phosphatases²³. Wip1, as a novel oncogene²⁴⁻²⁷, is currently believed to be a promising therapeutic target for cancers^{28, 29}. The involvement of Wip1 in the hematopoietic system recently caused great attentions. Wip1 critically regulates granulocyte development and function via p38MAPK-STAT1-dependent pathways³⁰⁻³³. It is known that Wip1 is essential for the homeostasis of mature medullary thymic epithelial cells, maturation of T cells in the thymus and antigen-independent B cell development from hematopoietic stem in both p53-dependent and independent manners³⁴⁻³⁶. However, it remains unknown how Wip1 controls CD4⁺ T cell polarization into Th1, Th2, Th9, Th17 and iTreg cells. In the present study, we used Wip1-deficient mice to address this issue. We found that Wip1 deficiency selectively impaired Th9 cell differentiation through a JNK-c-Jun/c-Fos pathway. Interestingly, the reduction in Th9 cell development leads to a decreased allergic response in an OVA-induced asthma mouse model demonstrating the importance of this subset for allergy development. Moreover, administration of a specific Wip1 inhibitor significantly reduces the pathology of allergic airway inflammation in mice. Thus, our study reveals a novel function of the phosphatase Wip1 in regulating Th9 cell development and underlines the potency of Wip1 as a molecular target for the treatment of Th9-mediated

189 allergic inflammation. Our findings pave the way for the development of new
190 and specific treatments of allergic asthma.
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Materials and Methods

Mice Wip1 knockout (KO) mice were provided by the Key Laboratory of Human Diseases Comparative Medicine, the Ministry of Public Health (Beijing, China). Wip1KO mice have been backcrossed to the C57BL/6 background in our laboratory^{30, 35}. C57BL/6 (B6) mice were purchased from Beijing University Experimental Animal Center (Beijing, China). Rag2KO were purchased from Beijing Huafukang Animal Center (Beijing, China). All mice were bred and maintained under specific pathogen-free conditions in the animal facility of Institute of Zoology, Chinese Academy of Science. Sex-matched littermate mice of 8-12 weeks were mainly used for experiments. All animal studies were approved by the Animal Ethics Committee of the Institute of Zoology (Beijing, China).

Differentiation of CD4⁺ T cell subpopulations from naïve T cells *in vitro*

Naïve T cells (CD4⁺CD44⁺CD62L⁺) were purified from spleens of WT or Wip1KO mice by MoFlo XDP (Beckman Coulter) or magnetic cell sorting (Stem cell). Naive CD4⁺ T cells (1×10^6 cells/ml in 96-well plates) were activated with plate-bound anti-CD3 (2 µg/ml; 145-2C11; R&D Systems) and soluble anti-CD28 (2 µg/ml; 37.51; BD Biosciences). At the same time, these cells were cultured under different Th cell induction conditions, respectively: Th9 induction conditions (IL-4: 20 ng/ml, R&D Systems; TGF-β: 2 ng/ml, R&D Systems; and anti-IFN-γ: 10 mg/ml, XMG1.2, eBioscience); Th2 induction conditions (IL-4: 20 ng/ml, R&D Systems; and anti-IFN-γ: 10 mg/ml, XMG1.2, eBioscience); Th1 induction conditions (IL-12: 10 ng/ml; PeproTech; IL-2: 100

U/ml, PeproTech; and anti-IL-4: 10 mg/ml, 11B11, eBioscience); Th17 induction conditions (TGF- β : 1 ng/ml, R&D Systems; IL-6: 50 ng/ml, R&D Systems; anti-IFN- γ : 10 mg/ml, XMG1.2, eBioscience; anti-IL-4: 10 mg/ml, 11B11, eBioscience; and anti-IL-2: 10 mg/ml, JES6-1A12, eBioscience); and Treg induction conditions (TGF- β : 1 ng/ml, R&D Systems; IL-2: 100 U/ml, PeproTech). After 3 days or the indicated time points of differentiation, cell polarization was assessed by intracellular staining and real-time PCR.

Flow cytometry After 3-5 days of culture, cells were stimulated with PMA (phorbol 12-myristate 13-acetate; 50 ng/ml), ionomycin (500 ng/ml; Sigma-Aldrich) for 5 h at 37 °C and Golgi-stop (BD Pharmingen) was added to the cells for the last 3h of stimulation. Intracellular cytokine staining was carried out with Fixation/Permeabilization buffer solution according to the manufacturer's protocol (BD Bioscience). Fluorochrome-conjugated anti-mouse IL-9 (RM9A4; BioLegend), anti-mouse IL-4 (11B11; eBioscience), anti-mouse IL-13 (eBio13A, eBioscience), anti-mouse IL-17A (TC11; Biolegend), anti-mouse IFN- γ (XMG1.2; Biolegend), anti-mouse TNF- α (MP6-XT22, Biolegend), anti-mouse PU.1 (7C2C34, Biolegend), anti-mouse IRF4 (IRF4.3E4, Biolegend), anti-mouse T-bet (4B10, Biolegend) and anti-mouse Foxp3 (NRRF-30; eBioscience) were used for staining. Foxp3, PU.1, IRF4, GATA3, T-bet staining was carried out with Fixation/Permeabilization buffer solution according to the manufacturer's protocol (eBioscience). Stained cells were analyzed on a FACSCalibur (BD Bioscience) or Epics XL (Beckman Coulter) with FCS Express 3 software.

Real-time PCR Total RNA was extracted from cultured cells with a total RNA kit (Promega), and cDNA was synthesized with cDNA Reverse Transcription kit (Takara). Quantitative real-time PCR was performed on the CFX96 real-time system (Bio-Rad) using Power SYBR® PCR Master Mix (Applied Biosystems). All real-time PCR reactions were carried out using optical 96-well reaction plate and run in triplicate ³¹. Each plate included two no template controls (NTCs). The primers used in the present study are summarized in Table 1. The real-time PCR data were analyzed by using comparative Ct method and were normalized using the housekeeping gene HPRT.

ELISA IL-9 in cell culture supernatants and homogenate of lung tissues was measured with ELISA kit according to the manufacturer's instructions (eBioscience). Lung tissue (200 mg) was homogenized in 1 ml RIPA buffer, centrifuged (3,000 rpm, 10 min) and the supernatant collected. IL-17, TNF- α , IL-4, IL-5, IL-13, MCP-1 were detected by ELISA kits of IL-17 (Biolegend), TNF- α (Biolegend), IL-4 (Biolegend), IL-5 (Biolegend), IL-13 (eBioscience) and MCP-1 (eBioscience) according the manufacturer's instructions. Mouse OVA-specific IgG, IgG2b, and IgG1 in sera were detected by quantitative ELISA kit of mouse anti-OVA IgG (Chondrex), mouse anti-OVA IgG2b (Chondrex) and mouse anti-OVA IgG1 (Chondrex), according the manufacturer's instructions.

Induction of allergic airway inflammation Allergic airway inflammation was induced as described ³⁷. Mice of 8-10 weeks were sensitized by intraperitoneal injection of ovalbumin (OVA, Sigma-Aldrich) adsorbed with

aluminum hydroxide (Sigma-Aldrich) at a dose of 10 µg OVA per 1 mg aluminum hydroxide (200 µl) on days 0 and 14. From day 21, mice were exposed to aerosolized OVA (1% w/v) for 30 min per day for 5 consecutive days. Mice were killed 2 days after the final intranasal challenge.

For studies with adoptive transfer of CD4⁺ T cells, 1×10^7 sorted CD4⁺ T cells of WT and Wip1KO mice were adoptively transferred to Rag2KO mice respectively by i.v. injection by one day before OVA sensitization. These mice were then conducted OVA-induced allergic airway inflammation as described above.

To evaluate the effect of Wip1 inhibitor (CCT007093, Sigma-Aldrich) on mouse allergic airway inflammation, Wip1 inhibitor (2.5 mg/ml, 25 µl per mouse) or with the same dose of dimethyl sulfoxide (DMSO) was intraperitoneal injected to mice daily from day 20 to day 24 during allergic airway inflammation induction phase.

The trachea was cannulated and lungs were lavaged with 3 ml PBS. Cell numbers in BAL fluid were counted with a hemocytometer. Cells in BAL fluid were analyzed by flow cytometry with antibodies of CD11b (M1/70, eBioscience), Siglec F (E50-2440, BD Pharmingen), Ly6G (1-A8, BD Biosciences), CD3e (145-2C11, eBioscience), B220 (RA3-6B2, Biolegend) and F4/80 (BM8, eBioscience) analyzed. RNA was isolated from cells in BAL fluid for real-time PCR analysis. Peripheral immune responses in sensitized and challenged mice were assessed by culture of splenocytes for 72 h with OVA (100 µg/ml). Lung tissue samples were prepared from control mice and

mice in OVA-induced allergy inflammation, embedded in paraffin and sectioned, then stained with hematoxylin and eosin (H&E) and PAS for evaluation of the infiltration of inflammatory cells and mucin production in trachea.

Airway hyperresponsiveness For airway hyperresponsiveness detection, a FinePointe NAM system (Buxco Research Systems) were used. In the FinePointe NAM system, sRaw (specific airway resistance) values represent airway hyperresponsiveness and lung functions of mice. Allergic airway inflammation was induced in mice as described above. 48 hrs after the last allergen challenge, WT and Wip1KO mice were placed in the NAM plethysmograph, and received nebulized PBS or increasing concentrations of methacholine (5, 10, 20, and 40 mg/ml) for 1 min. After the mice respond to them for 1 min, the sRaw values were measured for 2 min. The results were showed as the fold increase relative to the sRaw value (baseline) before mice were treated with nebulized PBS or methacholine.

Immunoblot Naïve CD4⁺ T cells were cultured in 1640 medium with 10% FBS. After stimulation, cells were washed once in cold PBS and lysed in RIPA buffer (50 mM Tris–HCl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA pH 7.4) with protease and phosphatase inhibitor cocktails (Roche) for 10 min on ice. Protein concentration was determined using a BCA assay³⁸. The lysates were mix with 5× SDS-PAGE loading buffer and boiled at 100°C for 5 min. Protein samples were size-fraction ated on 10% SDS–PAGE and transferred onto PVDF membranes (Millipore, CA). After blocked for 1 h,

then the membranes were incubated with primary antibodies overnight at 4°C. The appropriate HRP-conjugated secondary antibody was then added, and was detected through chemiluminescence (Millipore). Actin was used as a protein loading control. Phospho-STAT6 (Tyr641), STAT6, phospho-Smad3 (Ser423/425), Smad3, phospho-p38 MAPK (Thr180/Tyr182), P38, p-STAT5 (Tyr694), STAT5, p-STAT1 (Ser727), STAT1, phospho-SAPK/JNK (Thr183/Tyr185), SAPK/JNK, phospho-c-Jun (Ser73), c-Jun, Rel-B, NF- κ B p65, β -Actin and histone H3 were purchased from CST company.

Luciferase assay Luciferase reporter assays were done as reported³⁹. The mouse *Il-9* promoter region (-2162 to +28) was amplified from genomic DNA by PCR with forward primer (5'-TCGacgcgtGATGACCTGTGACCTGAC-3') and reverse primer (5'-TCCcccgggCAGAGGCAAGGATGTATGT-3'). Lowercase letters in primers were Mlu I and Sma I sites introduced to allow forced cloning into the pGL3 basic luciferase reporter gene vector. HEK293T cells were transfected with the reporter vector together with expression plasmids containing cDNA encoding mouse c-Jun and c-Fos and the pRL-TK vector (Promega) as an internal control through the use of Lipofectamine 2000 method (Invitrogen). After 24h, *Il-9* promoter activity was assessed by a dual-luciferase reporter assay system according to the manufacturer's instructions (Promega). All samples were normalized for transfection efficiency by division of firefly luciferase activity by renilla luciferase activity and the assays were repeated three times.

ChIP assay ChIP assay were carried out as described⁴⁰. WT and Wip1KO

CD4⁺CD44⁻CD62L⁺ T cells were purified by magnetic cell sorting (Stem Cell) and were cultured at a density of 1×10^6 cells per well. After stimulation in Th9-inducing condition for 3 days, cells were collected and then were fixed for 10 min at room temperature with 1% formaldehyde (Sigma-Aldrich) and lysed in lysis buffer. Lysates were sonicated, followed by precipitation with anti-c-Jun (Abcam), control rabbit IgG (Abcam), anti-PU.1 (CST) and anti-IRF4 (CST). Immunoprecipitated DNA was reverse-cross-linked, extracted with phenol and chloroform and then analyzed by real-time PCR with *Il-9*-c-Jun forward primer (5'-AGAACCCGACTATTTGAAGAGCATC-3') and reverse primer (5'-CTCAGTCTACCAGCATCTTCCA-3'), *Il-9*-PU.1 forward primer (5'-GTCACTTGACAAAGGCTGTCTTATGCC-3') and reverse primer (5'-GTCACTTGACAAAGGCTGTCTTATGCC-3'), and *Il-9*-IRF4 forward primer (5'-GATTCTCACAACCAGAATTCCTGC-3') and reverse primer (5'-CTACCAGCATCTTCCAGTCTAGC-3').

Statistical analysis All data were presented as mean $\pm$ SD. Unpaired two-tailed Student's *t*-test was used for data of two groups. Data more than two groups were subjected to one-way analysis of variance (ANOVA) with SAS 9.2 version. Comparisons were considered statistically significant when the *P* value was less than 0.05. **P* < .05, ** *P* < .01, *** *P* < .001.

Results

Wip1 deficiency does not impact Th1, Th2 and Th17 cell differentiation

To investigate the involvement of Wip1 in Th cell subtypes development, we employed Wip1KO mice and firstly analyzed whether Wip1 is required for naïve T cell activation and proliferation. The sorted naïve CD4⁺ T cells from WT and Wip1-deficient mice were activated with anti-CD3/anti-CD28 mAbs *in vitro*. As shown in Figure 1, naïve Wip1-deficient CD4⁺ T cells proliferated as efficient as WT cells after activation as determined by CFSE dilution assays (Fig 1, A). Consistently, naïve WT and Wip1KO CD4⁺ T cells showed comparable up-regulation of activation markers like CD25 and CD69 molecules after stimulation with anti-CD3/anti-CD28 mAbs (Fig 1, B and C). Thus, these data indicate that Wip1 is not required for naïve CD4⁺ T cell activation in terms of cell proliferation and activation marker expressions.

Next, we explored the potential roles of Wip1 in CD4⁺ T cell polarization and differentiation by using the standard *in vitro* cytokine cocktails induction systems for distinct Th subsets. Naïve Wip1-deficient CD4⁺ T cells differentiated into Th1, Th2 and Th17 cells as efficiently as naïve WT CD4⁺ T cells in the corresponding culture systems, respectively, as detected by real-time PCR and intracellular cytokine staining with flow cytometry (Fig 1, D-I). Furthermore, similar amounts of IL-4, IL-5 and IL-13 in the culture medium of Th2-polarized WT and Wip1-deficient CD4⁺ T cells were detected (Fig E1). On the other hand, very few of CD4⁺ T cells (< 2%) became IL-9⁺ cells and very low levels of IL-9 in the medium were detected in the Th2 induction system (Fig E2). No detectable impact of Wip1 on the IL-9

expression was observed in the Th2 induction system of naïve WT and Wip1-deficient CD4⁺ T cells (Fig E2). However, we found slightly but significantly higher *Foxp3* mRNA expression in naïve Wip1-deficient CD4⁺ T cells when induced for CD4⁺Foxp3⁺ iTreg differentiation as compared with naïve WT CD4⁺ T cells (Fig 1, J). These observations were further supported by the increased percentage of Foxp3⁺ T cells in Wip1-deficient CD4⁺ T cells than those in WT CD4⁺ T cells under iTreg induction conditions (Fig 1, K). Therefore, Wip1 deficiency does not intrinsically influence Th1, Th2 and Th17 cell differentiation *in vitro* despite a slight increase of *Foxp3* expression under iTreg induction.

Wip1 controls Th9 cell development

Recently, Th9 cells are well defined by secreting large amounts of IL-9 but less IL-4, IL-5 and IL-13 upon activation and have been shown to promote allergic airway inflammation progression^{19, 41}. Thus, we examined the roles of Wip1 in Th9 cell differentiation. WT CD4⁺ T cells expressed high level of *Il-9* as detected by real-time PCR (Fig 2, A) when activated with anti-CD3/anti-CD28 mAbs in the presence of TGF- β and different doses of IL-4 to induce Th9 cells, as reported previously⁴². However, Wip1-deficient CD4⁺ T cells expressed remarkably less *Il-9* mRNA than WT CD4⁺ T cells under the same polarization setting (Fig 2, A). The decreased IL-9 expression at the mRNA level in the induced Wip1-deficient CD4⁺ T cells was further confirmed by the reduced concentrations of IL-9 in the culture medium as indicated by ELISA (Fig 2, B). Consistently, we also observed the decreased frequency of IL-9⁺ T cells in Wip1-deficient CD4⁺ T cells as determined with flow cytometry assays (Fig 2,

C). Furthermore, during Th9 differentiation *in vitro*, the lower expression levels of *Il-9* in Wip1-deficient CD4⁺ T cells were observed in all culture time points (Fig 2, D), excluding the possibility that the decreased *Il-9* expression is simply due to the altered response kinetics. As expected, WT Th9 cells expressed extremely low levels of Th2 cytokines like *Il-4*, *Il-5*, and *Il-13* as compared to Th2 cells, and Wip1-deficient Th9 cells expressed lower Th2 cytokines (Fig E3). On the other hand, we observed similarly low levels of Foxp3⁺ T cells when WT and Wip1KO naïve CD4⁺ T cells were cultured in the Th9-inducing system (Fig 2, E). The low level expression of IL-4, IL-5, IL-13 and Foxp3 in Th9-inducing Wip1KO CD4⁺ T cells indicated that the poor Th9 differentiation in the absence of Wip1 is not due to the skewed differentiation into either IL-4-induced Th2 cells or TGF- β -induced Foxp3⁺ T cells. It is known that CCT007093 is a Wip1-specific inhibitor⁴³⁻⁴⁵. We thus examined the effects of CCT007093 on Th9 cell differentiation *in vitro*. Consistent with impaired Th9 differentiation of Wip1-deficient CD4⁺ T cells, Wip1 specific inhibitor CCT007093 significantly inhibited *Il-9* mRNA expression in WT CD4⁺ T cells in a dose-dependent manner (Fig 2, F). However, CCT007093 failed to further inhibit *Il-9* mRNA expression in Wip1KO CD4⁺ T cells (Fig 2, F), indicating that the inhibitory effect of CCT007093 on *Il-9* expression in CD4⁺ T cells is mainly mediated via a Wip1-dependent pathway. Furthermore, we show that CCT007093 treatment significantly decreases the percentage of CD4⁺IL-9⁺ T cells when assessed with intracellular FACS staining (Fig 2, G). We also further confirmed that Wip1 inhibitor did not significantly influence Th1 and Th2 differentiation (Fig E4 and E5) and slightly promoted CD4⁺Foxp3⁺ iTreg differentiation during the polarization of naïve WT Th cells, which is similar as

the observation showing the effects of Wip1 on Th cell polarization in Wip1-deficient Th cells (Fig E6). Altogether, we conclude that Wip1 is selectively required for Th9 cell development in a cell autonomous manner.

Wip1 regulates Th9 cell development through JNK-c-Jun/c-Fos pathway

To understand the molecular mechanisms underlying impaired Th9 differentiation caused by Wip1 deficiency, we first examined the expressions of transcription factors PU.1 and IRF4, which play important roles in Th9 development^{12, 13}. Indeed, the induced WT and Wip1-deficient Th9 cells expressed similar levels of PU.1 and IRF4 as determined by flow cytometry (Fig E7). Furthermore, ChIP assays demonstrated that both PU.1 and IRF4 display similar binding ability to the *IL-9* gene promoter between WT and Wip1-deficient Th9 cells (Fig E7). We then screened the candidate molecules down-stream of Wip1 and the potential targets known to regulate Th9 cell development, by analyzing WT and Wip1KO Th9 cells with western blots and real-time PCR. Under Th9 induction, we noticed increased activation of Stat6, Stat5, Stat1, Smad3, p38MAPK, JNK, c-Jun, RelB and p65 molecules in WT naïve CD4⁺ T cells, as indicated by their protein phosphorylation and their localization into the nuclei (Fig 3, A and B). However, as compared to their WT counterparts, Wip1KO CD4⁺ T cells displayed enhanced activation of p38MAPK, Stat1, JNK and c-Jun but the activation of Stat6, Stat5, Smad3 and NF-κB pathway remains comparable during Th9 induction (Fig 3, A and B), indicating the potential involvement of p38MAPK, Stat1, JNK and c-Jun pathways in the decreased Th9 differentiation caused by Wip1 deficiency. Moreover, inhibition of p38MAPK and Stat1 by SB203580 and

463 methylthioadenosine (MTA), respectively³⁹, failed to rescue the poor
464 differentiation of Wip1KO Th9 cells (Fig E8), suggesting that the increased
465 p38MAPK and Stat1 activity in Wip1KO T cells might not be responsible for
466 reduced Th9 cell differentiation. SP600125, a widely used JNK inhibitor⁴⁶,
467 decreased JNK activation and c-Jun activation in WT and Wip1KO T cells
468 during Th9 induction (Fig 3, C). Intriguingly, SP600125 greatly rescued the
469 deficient Th9 differentiation in Wip1-deficient Th cells as measured by
470 real-time PCR and intracellular staining with flow cytometry assays (Fig 3, D
471 and E). These results reveal that Wip1 regulates Th9 cell differentiation
472 through its inhibition on JNK-dependent pathways. The *Il-9* promoter region
473 and two additional regions of conserved non-coding sequence upstream of the
474 promoter region form the cis- and trans-regulatory elements collectively
475 regulating *Il-9* gene expression^{12, 21}. Sequence and bioinformatics analysis
476 have identified the potential binding sites for transcription factors including
477 PU.1, IRF4, STATs, NFAT, GATA1, Smads and Notch as well as NF- κ B and
478 AP-1 in the promoter region of *Il-9*^{21, 22}. Therefore, we investigated whether
479 AP-1 can directly bind to *Il-9* gene promoter and regulate *Il-9* expression. With
480 ChIP assays, we noticed an enhanced c-Jun binding to *Il-9* promoter during
481 WT and Wip1KO Th9 induction (Fig 3, F). There are two potential binding sites
482 for AP-1 located at -385 and -166 of *Il-9* promoter (Fig 3, G). In a luciferase
483 reporter system with HEK 293T cells, over-expression of c-Jun and c-Fos
484 significantly decreased *Il-9* promoter-driving luciferase activity in a
485 dose-dependent manner (Fig E9). Importantly, mutation of AP-1 binding site
486 located at -385 of the *Il-9* promoter (Mut2) completely reversed the inhibitory
487 effect of c-Jun/c-Fos on *Il-9* expression as indicated by the luciferase activity,

but mutation of the AP-1 binding site at -166 of *Il-9* promoter (Mut1) failed to do so (Fig 3, *H*). Thus, the AP-1 binding site at -385 of *Il-9* promoter is essential for the inhibitory role of AP-1 on *Il-9* expression. On the other hand, Wip1KO CD4⁺ T cells expressed similar levels of GATA3, BATF and Bcl6, which are also important for Th9 differentiation⁴⁷⁻⁴⁹, as compared to WT CD4⁺ T cells (Fig E7). Altogether, we conclude that Wip1 deficiency inhibits *Il-9* transcription via enhanced c-Jun/c-Fos binding to the promoter region.

Wip1KO mice show reduced allergic airway response

We next employed an OVA-induced allergic airway inflammatory mouse model, in which Th9 cells play a critical role as reported recently^{18, 19, 42, 50}. The airway hyperresponsiveness of WT and Wip1KO mice suffering from OVA-induced allergic airway inflammation was detected using a FinePointe NAM system⁵¹. Compared to WT mice, Wip1KO mice demonstrated significantly less airway hyperresponsiveness in response to methacholine challenge (Fig 4, *A*). We found significantly higher levels of OVA-specific IgE, OVA-specific IgG1 and OVA-specific IgG in the sera of WT littermates after OVA induced allergic response as determined by ELISA assays (Fig 4, *B*; Fig E10), which is consistent with the previous reports⁵⁰. However, Wip1KO mice have lower OVA-specific IgE, OVA-specific IgG1 and OVA-specific IgG productions after similar OVA induction (Fig 4, *B*; Fig E10). There were no detectable differences regarding OVA-specific IgG2b levels between WT and Wip1KO mice (Fig E10), which is mainly regulated by Th1 cells. Mucus production in bronchus of lung tissues reflects the severity of allergic inflammation and can be evaluated by Periodic acid Schiff reagent (PAS) staining⁵². Wip1KO mice produced less

513 mucus in lung tissues as compared with WT mice in OVA-induced allergic
514 airway inflammatory model, which was indicated by arrows (Fig 4, C).
515 Consistently, the mRNA expression of *Muc5ac*, which is associated with
516 mucus hypersecretion^{41, 53}, in lung tissues of OVA-induced Wip1KO mice was
517 significantly lower than that of WT mice (Fig 4, D). Mast cells also play
518 important roles in allergic airway inflammation and mouse mast cell protease 1
519 (MCP-1) levels in sera reflect the infiltration of mast cells in trachea⁴¹. We
520 found that the MCP-1 levels in sera and *Mcpt1* mRNA expression in lung
521 tissues were significantly lower in OVA-induced allergic airway inflammatory
522 Wip1KO mice as compared to WT counterparts (Fig 4, E; Fig E11).
523 Hematoxylin and eosin (H&E) staining showed that Wip1KO mice suffered less
524 severe pathogenesis of allergic inflammation in the lung tissues than WT mice
525 as indicated by the reduced pathological changes (Fig 4, F). The resistance to
526 OVA-induced allergic airway inflammatory response of Wip1KO mice was
527 further supported by the significant less infiltration by leukocytes and
528 eosinophils in the BAL fluid after OVA challenge (Fig 4, G and H). Moreover,
529 the freshly isolated cells from the BAL fluid and spleens of allergic airway
530 inflammatory Wip1KO mice expressed significantly less *Il-9*, as detected by
531 quantitative PCR (Fig 4, I and J). Freshly isolated CD4⁺ T cells from spleens of
532 OVA-challenged Wip1KO mice expressed less IL-9 molecules than CD4⁺ T
533 cells from OVA-challenged WT mice after be stimulated with OVA *in vitro* (Fig 4,
534 K). However, the cells isolated from the BAL fluid of Wip1KO mice with allergic
535 airway inflammation expressed similar levels of IL-4, IL-5, IL-13, IFN- γ and
536 IL-17 as those of WT mice with allergic airway inflammation, as detected by
537 quantitative PCR (Fig E12). Thus, deficiency of Wip1 impairs Th9 polarization

in vivo and reduces Th9 cell-mediated allergic airway inflammation.

Wip1 deficiency in CD4⁺ T cells impacts the allergic airway inflammation in mice

To determine whether Wip1 deficiency in CD4⁺ T cells alone is sufficient for the alleviative allergic immune response in an OVA-induced asthma mouse model, we assessed the severity of allergic airway inflammation in Rag2KO mice that were adoptively transferred with the sorted Wip1KO or WT CD4⁺ T cells before mice were sensitized for allergic airway inflammation induction. We observed similar levels of CD4⁺ T cells in the peripheral blood of Rag2KO mice with transfer of the sorted WT or Wip1KO CD4⁺ T cells (Fig 5, A), indicating that Wip1 deficiency does not impact the reconstitution ability of CD4⁺ T cells after adoptive transfer to immune-deficient recipients. Rag2KO mice receiving Wip1KO CD4⁺ T cells suffered less severe pathogenesis of allergic airway inflammation in lung tissues than mice with WT CD4⁺ T cell transfer as indicated by the reduced pathological changes (Fig 5, B). The reduced OVA-induced allergic inflammatory response with Wip1KO CD4⁺ T cell transfer was further supported by less infiltration by leukocytes and eosinophils in the BAL fluid after OVA challenge (Fig 5, C and D). Furthermore, Rag2KO mice receiving Wip1KO CD4⁺ T cells produced less mucus in lung tissues as compared to their counterparts harboring WT CD4⁺ T cells, as evaluated by PAS staining and *Muc5ac* mRNA expression in lung tissues (Fig 5, E and F). Meanwhile, the serum MCP-1 levels and the mRNA expression of *Mcpt1* in lung tissues of allergic inflammatory Rag2KO mice receiving Wip1KO CD4⁺ T cells were significantly lower than those Rag2KO mice transferred with WT

CD4⁺ T cells (Fig 5, G; Fig E13). Moreover, the levels of IL-9 in lung tissues of Rag2KO mice receiving Wip1KO CD4⁺ T cells were significantly lower as detected by ELISA (Fig 5, H). Freshly isolated CD4⁺ T cells from draining lymph nodes (dLNs) of OVA-challenged Rag2KO mice receiving Wip1KO CD4⁺ T cells expressed less IL-9 than CD4⁺ T cells from Rag2KO mice harboring WT CD4⁺ T cells (Fig 5, I). Furthermore, we observed reduced amount of secreted IL-9 in the culture supernatants of dLNs cells from Rag2KO mice receiving Wip1KO CD4⁺ T cells (Fig 5, J). However, the cells isolated from the BAL fluid of Wip1KO CD4⁺ T cells-reconstituted Rag2KO mice expressed similar levels of IL-4, IL-5 and IL-13 compared to those of mice received WT CD4⁺ T cells as detected by quantitative PCR (Fig E14). Thus, Wip1 deficiency in CD4⁺ T cells is sufficient to impair the allergic immune response in mice, which is mainly mediated by IL-9 in our model, although IL-4, IL-5 and IL-13 are also involved in the pathogenesis of allergic airway inflammation as reported previously⁵⁴⁻⁵⁷.

Wip1 inhibitor decreased the severity of allergic airway inflammation in mice

Given the fact that Wip1KO mice and Rag2KO mice receiving Wip1KO CD4⁺ T cells exhibit the resistance to allergic immune response, we thus explored whether inhibition of Wip1 by CCT007093^{44, 45} could block the allergic pathogenesis in an OVA-induced allergic airway inflammatory mouse model. CCT007093 is a Wip1-specific inhibitor to suppress tumor cell growth and promote liver regeneration *in vivo*^{44, 58, 59}. It inhibits Wip1 activity specifically and subsequently increased the phosphorylation of the known Wip1 target

molecules^{43, 60}. Administration of Wip1 inhibitor for 5 days during induction stage could significantly reduce the severity of allergic inflammation in WT mice as evidenced by the reduced pathological changes (Fig 6, A). The reduced allergic inflammation was further confirmed by much less infiltration of leukocytes and eosinophils into the BAL fluid (Fig 6, B and C) and significantly lowers OVA-specific IgE levels in the sera (Fig 6, D) in the Wip1 inhibitor-treated mice. In addition, PAS staining and *Muc5ac* mRNA expression of lung tissues showed that mice treated with Wip1 inhibitor CCT007093 produced less mucus in bronchus of lung tissues (Fig 6, E and F). The serum MCP-1 levels and *Mcpt1* mRNA expression in lung tissues were significantly decreased in mice treated with Wip1 inhibitor (Fig 6, G; Fig E15). The mRNA expression of *Il-9* was significantly decreased in the cells of BAL fluid after Wip1 inhibitor CCT007093 treatment (Fig 6, H). Meanwhile, the homogenate of lung tissues of Wip1 inhibitor-treated allergic inflammatory mice expressed significantly less IL-9, as detected by ELISA (Fig 6, I). We also observed reduced frequency of freshly isolated CD4⁺ T cells expressing IL-9 from dLNs of CCT007093-treated allergic inflammatory mice (Fig 6, J). In addition, CCT007093 treatment did not affect IL-4, IL-5, IL-13, IFN- γ , IL-17 and TNF- α expressions (Fig E16). CCT007093 treatment did not influence CD4⁺Foxp3⁺ Treg cells and T-bet⁺ Th1 cells in OVA-induced allergic airway inflammatory mice as indicated by the intracellular staining flow cytometry assays and the mRNA expressions of *Foxp3* and *Tbx21* in lung tissues and dLNs cells (Fig E17). Taken together, inhibition of Wip1 by CCT007093 significantly ameliorates the pathogenesis of allergic airway inflammation in mice.

Discussion

Asthma is one of the most common chronic diseases affecting millions of people worldwide. Current clinical therapies based on inhaled corticosteroids and long-acting β 2 agonists are effective in controlling asthma in most, but not all patients¹. We herein found that the treatment with phosphatase Wip1 inhibitor CCT007093⁴⁵, which has been proved to inhibit proliferation of several types of cancer cells, significantly decreased the pathogenesis of allergic airway inflammation in a mouse model. Wip1 inhibition significantly reduced pathological changes of the lungs, the eosinophils infiltration in the BAL fluid, the mucus in bronchus of lung tissues and the OVA-specific IgE levels in the sera of asthma-induced mice. The role of Wip1 in allergic airway inflammation was further confirmed with Wip1-deficient mice. Thus, Wip1 inhibitors may have potential application to treat allergic inflammatory diseases.

Asthma is a chronic inflammatory disease of the airways characterized by infiltration of eosinophils, basophils, mast cells, and CD4 Th cells into the airway submucosa, which are important cells underlying allergic eosinophilic asthma^{2, 3}. The role of IL-9 in allergic asthma is currently recognized. IL-9 expression is higher in lungs of asthmatic patients and transgenic expression of IL-9 results in allergic inflammation^{5, 6, 8, 9}. IL-9 promotes mucus production from lung epithelial cells and pulmonary mastocytosis^{10, 11}. However, it is also reported that IL-9 might not be involved in the allergic airway inflammation in mice¹⁶. The inconsistency is unclear right now. Our present studies show that Wip1 selectively impacts Th9 cell differentiation and deficiencies of Wip1 by

genetic modification or a chemical inhibitor reduce the pathogenesis of allergic airway inflammation in mice, indicating that Th9 cell subpopulation might contribute to the pathological changes of allergic asthma. This speculation is in line with a recent study showing that Th9 cells were involved in the lung allergic inflammation¹². Adoptive transferred Th9 cells lead to allergic airway disease characterized by increased amounts of eosinophil recruitment, tissue mast cell numbers, and serum IgE following OVA challenge^{13-15, 42}. All these data support the contribution of Th9 cells to allergic airway inflammation.

With the different Th cell subpopulation inducing systems, we found that Wip1 did not significantly impact the differentiation of Th1, Th2 and Th17 cells. Wip1 deficiency slightly but significantly increased the differentiation of CD4⁺CD25⁺ iTreg cells. However, either genetic deficiency or inhibition of Wip1 activity by its specific inhibitor selectively impaired Th9 cell differentiation. The poor Th9 differentiation of Wip1KO naïve CD4⁺ T cells is unlikely due to T cell activation failure, as sorted naïve CD4⁺ T cells proliferated efficiently and expressed comparable levels of activation markers like CD25 and CD69 upon anti-CD3/CD28 mAb stimulation as compared to WT naïve CD4⁺ T cells. Altogether, our observations support that phosphatase Wip1 intrinsically regulates Th9 cell differentiation.

Wip1KO naïve CD4⁺ T cells displayed higher activation of p38MAPK, Stat1, JNK and c-Jun than WT cells during Th9 induction as indicated by their protein phosphorylation and their localization into the nuclei, while WT and Wip1KO CD4⁺ T cells showed similar activation of Stat6, Stat5, Smad3 proteins and

NF- κ B pathway. However, specific inhibition of p38MAPK and Stat1 by SB203580 and MTA, respectively³⁹, failed to rescue the poor differentiation of Wip1KO Th9 cells, indicating that the increased p38MAPK and Stat1 activity in Wip1KO T cells is not responsible for impaired Th9 cell differentiation. PU.1 and IRF4 represent two important transcription factors for Th9 cell differentiation^{12, 13}. However, we did not observe any significant difference regarding either PU.1 and IRF4 expression or their binding to *IL-9* promoter between WT and Wip1KO Th9 cells. Therefore, PU.1 and IRF4 may not contribute to the defective differentiation of Th9 cells caused by Wip1 deficiency. Interestingly, JNK inhibitor SP600125 significantly decreased c-Jun activation and rescued the deficiency of Wip1KO Th9 differentiation. These results suggest that Wip1 may regulate Th9 cell differentiation through JNK-dependent pathway. Our ChIP assays further reveal enhanced c-Jun binding to *IL-9* promoter in Wip1KO Th9 cells, which are in line with the previous reports showing the potential binding sites of *IL-9* promoter region for transcription factor AP-1^{21, 22}. Furthermore, over-expression of c-Jun and c-Fos significantly decreased *IL-9* promoter-driving luciferase activity in a dose-dependent manner. We further demonstrate that the Ap-1 binding site at -385 of *IL-9* promoter is essential for inhibiting *IL-9* expression. On the other hand, Wip1KO CD4⁺ T cells expressed comparable levels of GATA3, BATF and Bcl6, which are also important for Th9 induction⁴⁷⁻⁴⁹, as WT CD4⁺ T cells during Th9 induction. Collectively, we conclude that phosphatase Wip1 deficiency reduces *IL-9* gene transcription via enhanced binding of c-Jun/c-Fos to the promoter, which is activated by JNK. These findings shed lights on a previously unrecognized role of Wip1 in Th9 cell differentiation.

689

690 Wip1, as a novel oncogene²⁴⁻²⁷, is believed as a promising therapeutic target
691 for cancers^{28, 29}. It was recently reported that Wip1 inhibitor CCT007093 could
692 promote liver regeneration in mice⁴⁴. In the present study, we show that
693 CCT007093 significantly decreases allergic airway inflammation and
694 Wip1-deficient mice are resistant to allergic airway inflammation, indicating
695 that Wip1 may be essential for the sensitization and induction of allergic airway
696 inflammation in OVA-induced asthma mice. However, the Wip1 inhibitor
697 CCT007093 was given during allergic airway inflammation induction but not
698 during mouse sensitization by OVA immunization, thus, Wip1 inhibitors may be
699 potentially used to treat allergic airway inflammation.

700

701 Th2 cells are well recognized to be involved in allergic airway inflammation^{61, 62}.
702 Recently, accumulating evidences suggest that Th9 cells and IL-9 may also
703 play a critical role in allergic airway inflammation, although it remains debated⁷,
704 13, 16, 18, 63. Those discrepancies may be related to different animal models,
705 treatment approaches or distinct time points. It may also reflect the complexity
706 regarding Th9 cells in allergic inflammation. With an adoptive transfer mouse
707 model in which Rag2KO mice received either WT or Wip1KO CD4⁺ T cells, we
708 present the data that Wip1 deficient Th9 cells ameliorates the pathogenesis of
709 allergic airway inflammation. It remains to be investigated regarding the key
710 players for the decreased allergic airway inflammation mediated by
711 Wip1-deficient Th9 cells in mice. It is likely that IL-9 produced by Th9 cells is
712 the main mediator in our model, the involvement of other cytokines such as
713 IL-13 in this process need to be further determined.

714

715 In summary, we have uncovered a novel role of Wip1 in regulating Th9 cell
716 development through a JNK-dependent c-Jun/c-Fos-mediated direct inhibition
717 on *Il-9* transcription. Our findings that Wip1 inhibitor significantly decreased the
718 progression of mouse allergic airway inflammation may have important
719 implications for clinical application of Wip1 inhibitors in allergy therapies.

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Author contributions

P.W. designed and carried out the experiments, analyzed data and wrote the manuscript; H.S. and H.C. carried out the experiments, and analyzed data; L.Z. (Lianjun Zhang) analyzed data and wrote the manuscript; X.H. Breed mice and performed PCR assays; F.Y. genotyped the genetically modified mice; J.L. analyzed data, revised the manuscript and provided comments; L.Z. (Lianfeng Zhang) provided animal models and revised the manuscript; Y.Z. designed experiments, analyzed data, wrote the manuscript and provided overall supervision.

Competing financial interests

The authors have no competing financial interests.

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Figure legends

Figure 1. The activation and differentiation of Th cell subpopulations of WT and Wip1KO naïve CD4⁺ T cells.

(A) CFSE dilution in WT and Wip1KO CD4⁺ T cells stimulated for 72h with plate-bounded anti-CD3 and soluble anti-CD28. The surface expression of activation associated marks CD25 (B) and CD69 (C) of WT and Wip1KO naïve CD4⁺ T cells stimulated for 72h with plate-bounded anti-CD3 and soluble anti-CD28. (D) *Ifng* mRNA expression in the induced Th1 cells of WT and Wip1KO mice, which were stimulated for 72h under Th1-skewing condition. (E) The percentage of CD4⁺IFN- γ ⁺ T cells in naïve CD4⁺ T cells stimulated for 5d under Th1-skewing condition. (F) Expression of *Il-4* mRNA in the induced Th2 cells of WT and Wip1KO mice stimulated for 72h under Th2-skewing condition. (G) The percentage of CD4⁺IL-4⁺ T cells in naïve CD4⁺ T cells cultured under Th2-skewing condition for 5d. (H) *Il-17* mRNA expression in Th17 cells of WT and Wip1KO mice under Th17-skewing condition for 72h. (I) The percentage of CD4⁺IL-17⁺ T cells in naïve CD4⁺ T cells cultured under Th17-skewing condition for 5d. (J) *Foxp3* mRNA expression in iTreg cells of WT and Wip1KO mice under iTreg-skewing condition for 48h. (K) Expression of iTreg-specific transcription factor Foxp3 after naïve CD4⁺ T cells stimulated for 3d under iTreg-skewing condition. Data are shown as mean $\pm$ SD ($n = 3$), representing one of three independent experiments. * $P < .05$.

965

966 **Figure 2. Wip1 is essential for Th9 cell differentiation *in vitro*.**

967 (A) Expression of *Il-9* mRNA in WT and Wip1KO naïve CD4⁺ T cells cultured
968 under Th9-skewing condition with different doses of IL-4 for 48h. (B) The
969 levels of IL-9 in the culture medium of WT and Wip1KO naïve CD4⁺ T cells
970 cultured under Th9-skewing condition as in A. (C) Intracellular staining of IL-9
971 in CD4⁺ T cells differentiated for 72h with anti-CD3 and anti-CD28 plus
972 medium alone or TGF- β plus different concentrations of IL-4. (D) Expression of
973 *Il-9* mRNA of naïve WT and Wip1KO CD4⁺ T cells during Th9 differentiation
974 (0-3d). (E) Intracellular staining of IL-9 and Foxp3 in CD4⁺ T cells cultured
975 under Th9-skewing condition for 72h. (F) Expression of *Il-9* mRNA of naïve WT
976 and Wip1KO CD4⁺ T cells cultured with Th9-skewing condition with various
977 concentrations of Wip1 inhibitor for 48h. (G) Intracellular staining of IL-9 in
978 CD4⁺ T cells differentiated for 72h under Th9-skewing condition with Wip1
979 inhibitor (5 μ M). Data are shown as mean $\pm$ SD ($n = 3$), representing one of
980 three independent experiments. * $P < .05$, ** $P < .01$, *** $P < .001$.

981

Figure 3. Wip1 regulates *Il-9* mRNA and protein expression in Th9 cells differentiation through inhibiting JNK-c-Jun/c-Fos pathway.

(A) Immunoblot analysis of p-Stat6, Stat6, p-Smad3, Smad3, p-p38, p38, p-Stat5, Stat5, p-Stat1, Stat1, p-JNK, JNK, p-c-Jun, c-Jun and β -actin in naïve CD4⁺CD44⁻CD62L⁺ cells from WT and Wip1KO mice polarized under Th9 conditions for the indicated time points (0-120 mins). (B) Immunoblot analysis of Rel-B, p65 and H3 in the nucleus proteins of naïve WT and Wip1KO CD4⁺ T cells left unstimulated or stimulated under Th0 or Th9 condition for 24h. (C) Immunoblot analysis of p-c-Jun, c-Jun, p-JNK, JNK and β -actin in naïve WT and Wip1KO CD4⁺ cells treated with (+) or without (-) JNK specific inhibitor (SP600125) under Th9-inducing condition for the indicated time points (0-15 mins). (D) *Il-9* mRNA expression of naïve WT and Wip1KO CD4⁺ cells treated with or without SP600125 under Th9-inducing condition for 48h. (E) Intracellular IL-9 expression was determined by flow cytometry. (F) Naïve WT and Wip1KO CD4⁺ T cells were stimulated under Th9-inducing condition for 3 days, and then a ChIP assay was performed using anti-c-Jun mAb as described in the methods. (G) The potential AP-1 binding sites in the *Il-9* promoter. (H) HEK 293T cells were transfected with active c-Jun and c-Fos together with WT or mutated *Il-9* promoter luciferase vector. The cells were lysed and luminescence was measured 24h later. Data are shown as mean $\pm$ SD ($n = 3$), representing one of at least two independent experiments. * $P < .05$, ** $P < .01$.

Figure 4. Wip1-deficient mice are resistant to allergic airway inflammation.

(A) The airway hyperresponsiveness of allergic airway inflammatory mice was examined using a FinePointe NAM system as described in materials and methods. The specific airway resistance represents airway hyperresponsiveness and lung functions of mice. The un-induced mice serve as control. (B) The levels of OVA-specific IgE in the sera of WT and Wip1KO mice with or without OVA-induced allergic airway inflammation (AAI) ($n = 4-6$ per group). (C) Periodic acid Schiff reagent (PAS) staining was performed on lung tissues of WT and Wip1KO mice 48h after the last OVA challenge. Original magnification, $\times 100$. (D) *Muc5ac* mRNA expression in lung tissues of WT and Wip1KO mice. (E) The mouse MCP-1 levels in sera of WT and Wip1KO mice. (F) H&E staining of lung tissues of WT and Wip1KO mice and cell numbers of WT and Wip1KO mice in BAL fluid. Original magnification, $\times 100$. (G) The percentages of eosinophils in BAL fluid of WT and Wip1KO were assessed by flow cytometry. (H) Quantification of cells in BAL fluid and lung infiltrates from WT and Wip1KO mice ($n = 4-6$ per group) in allergic asthma model. (I) *IL-9* mRNA expression in cells of BAL fluid isolated from WT and Wip1KO mice. (J) *IL-9* mRNA expression and (K) intracellular IL-9 staining in splenocytes of WT and Wip1KO mice ($n = 4-6$ per group) stimulated for 3 days with OVA. Data represent two or three independent experiments. Data are shown as mean $\pm$ SD, $**P < .01$.

Figure 5. Wip1 inhibitor significantly alleviates the pathogenesis of allergic airway inflammation *in vivo*.

(A) The presence of CD4⁺ T cells in Rag2KO mice received sorted CD4⁺ T cells (WT→Rag2KO) and Wip1KO CD4⁺ T cells (Wip1KO→Rag2KO). (B) Lung tissue H&E staining of allergic inflammatory Rag2KO mice received WT and Wip1KO CD4⁺ T cells. Original magnification, ×50 (top row) or ×100 (bottom row). (C) Total cell numbers and eosinophil numbers in BAL fluid of Rag2KO mice received WT and Wip1KO CD4⁺ T cells in allergic airway inflammation model (*n* =5 per group). (D) The percentages of eosinophils in BAL fluid of Rag2KO mice received WT and Wip1KO CD4⁺ T cells were assessed by flow cytometry. (E) PAS staining of lung tissues of Rag2KO mice received WT and Wip1KO CD4⁺ T cells in allergic airway inflammation model. Original magnification, ×50 (top row) or ×100 (bottom row). (F) *Muc5ac* mRNA expression in lung tissues of allergic airway inflammatory Rag2KO mice with WT and Wip1KO CD4⁺ T cell transfer. (G) The mouse MCP-1 levels in the sera of Rag2KO mice received WT and Wip1KO CD4⁺ T cells, respectively. (H) The protein levels of IL-9 in the homogenate of lung tissues of allergic Rag2KO mice received WT and Wip1KO CD4⁺ T cells as determined by ELISA. (I) The IL-9 expression of CD4⁺ T cells of dLNs of allergic inflammatory Rag2KO mice received WT and Wip1KO CD4⁺ T cells (*n* = 5 per group). (J) The protein levels of IL-9 in the culture medium of cells isolated from dLNs after stimulated with OVA for 3 days. Data (mean ± SD) represent one of two independent

experiments. $*P < .05$, $**P < .01$ and $***P < .001$.

Figure 6. Wip1 inhibitor significantly alleviates the pathogenesis of allergic airway inflammation *in vivo*.

(A) Lung tissue H&E staining of WT mice treated with buffer and Wip1 inhibitor in allergic airway inflammation model. Original magnification, $\times 50$ (top row) or $\times 100$ (bottom row). (B) Cell numbers in BAL fluid of mice treated with buffer or wip1 inhibitor in allergic airway inflammation model ($n = 4-6$ per group). (C) The percentages of eosinophils in BAL fluid of mice treated with buffer and Wip1 inhibitor. (D) OVA-specific IgE levels in the sera of allergic airway inflammatory mice treated with buffer and Wip1 inhibitor, respectively ($n = 4-6$ per group). (E) PAS staining of lung tissues of mice treated with Wip1 inhibitor in allergic airway inflammation model. Original magnification, $\times 50$ (top row) or $\times 100$ (bottom row). (F) *Muc5ac* mRNA expression in lung tissues of allergic airway inflammatory mice treated with buffer and Wip1 inhibitor. (G) The mouse MCP-1 levels in the sera of allergic airway inflammatory mice treated with buffer and Wip1 inhibitor, respectively. (H) *Il-9* mRNA expression of cells in BAL fluid of allergic airway inflammatory mice treated with buffer and Wip1 inhibitor. (I) The levels of IL-9 in the homogenate of lung tissues of mice treated with buffer and Wip1 inhibitor in allergic airway inflammation model. (J) The IL-9 expression of $CD4^+$ T cells of dLNs of allergic airway inflammatory mice treated with buffer and Wip1 inhibitor ($n = 4-6$ per group). Data (mean $\pm$

1072 SD) represent one of two or three independent experiments. * $P < .05$ and ** P

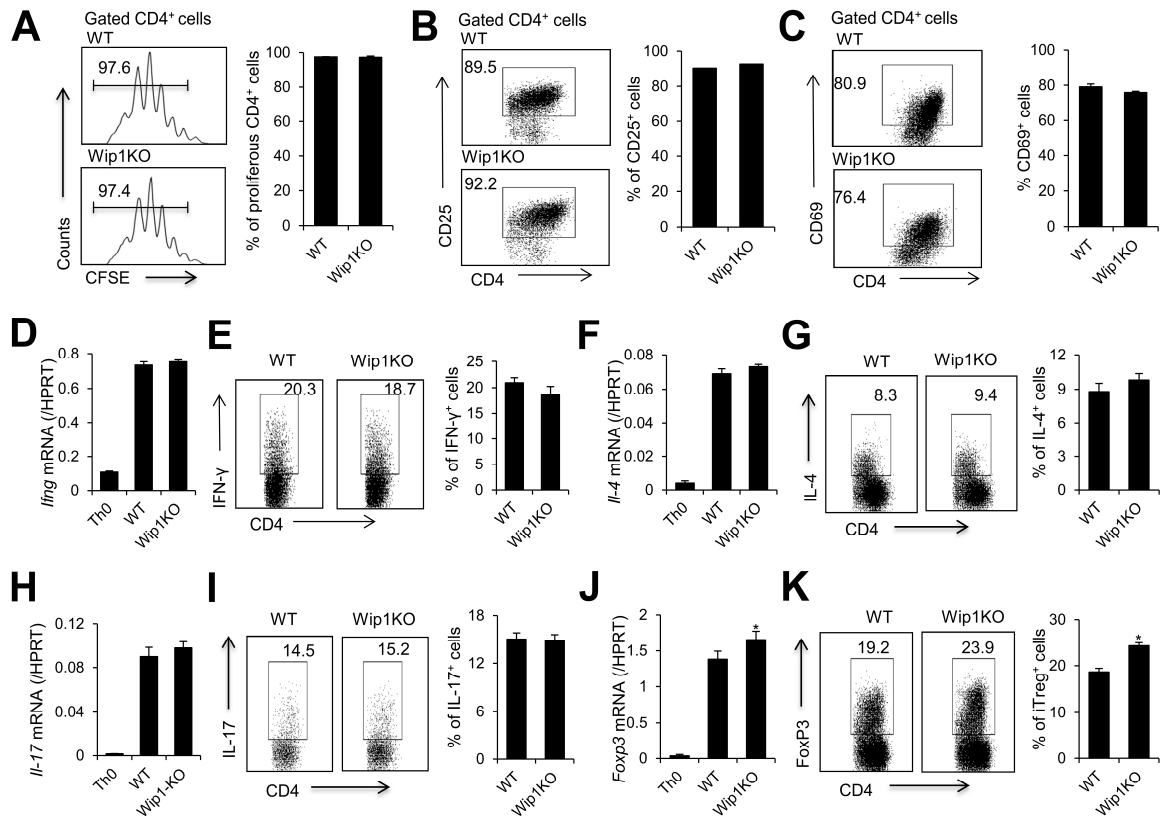
1073 $< .01$.

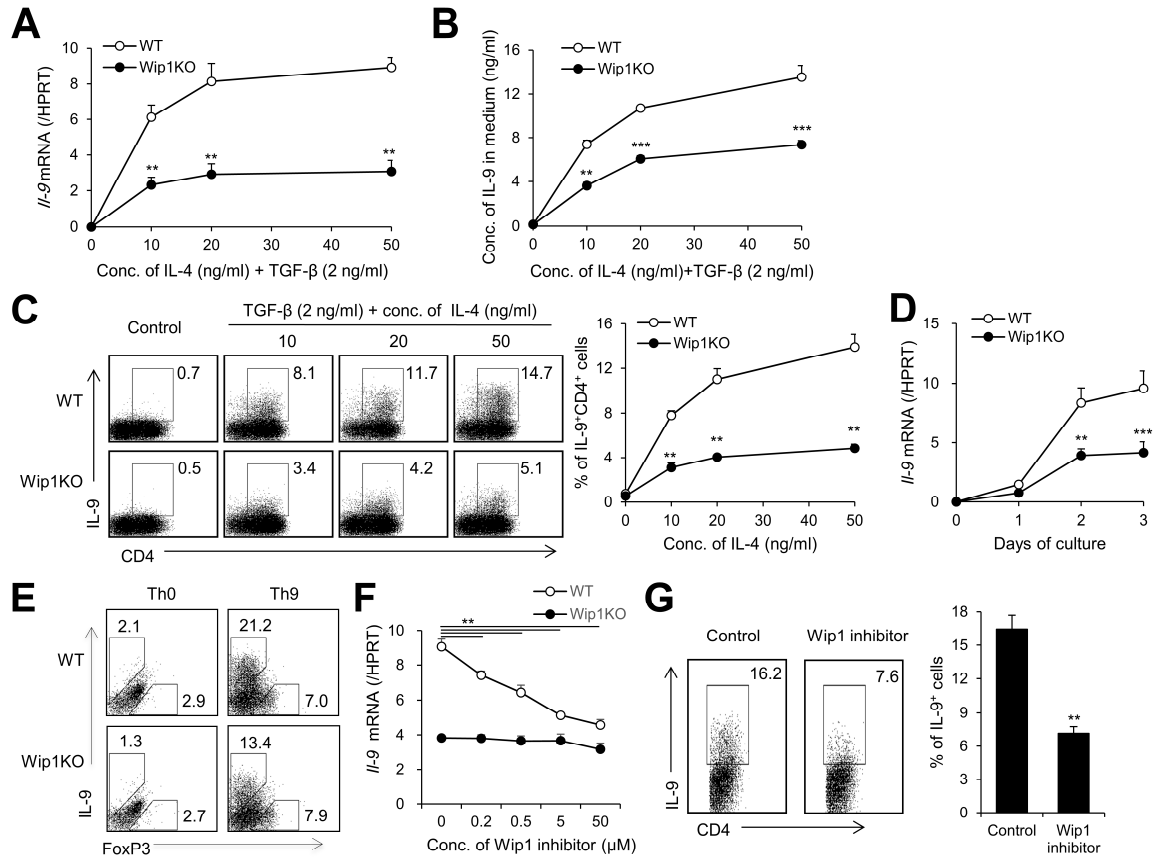
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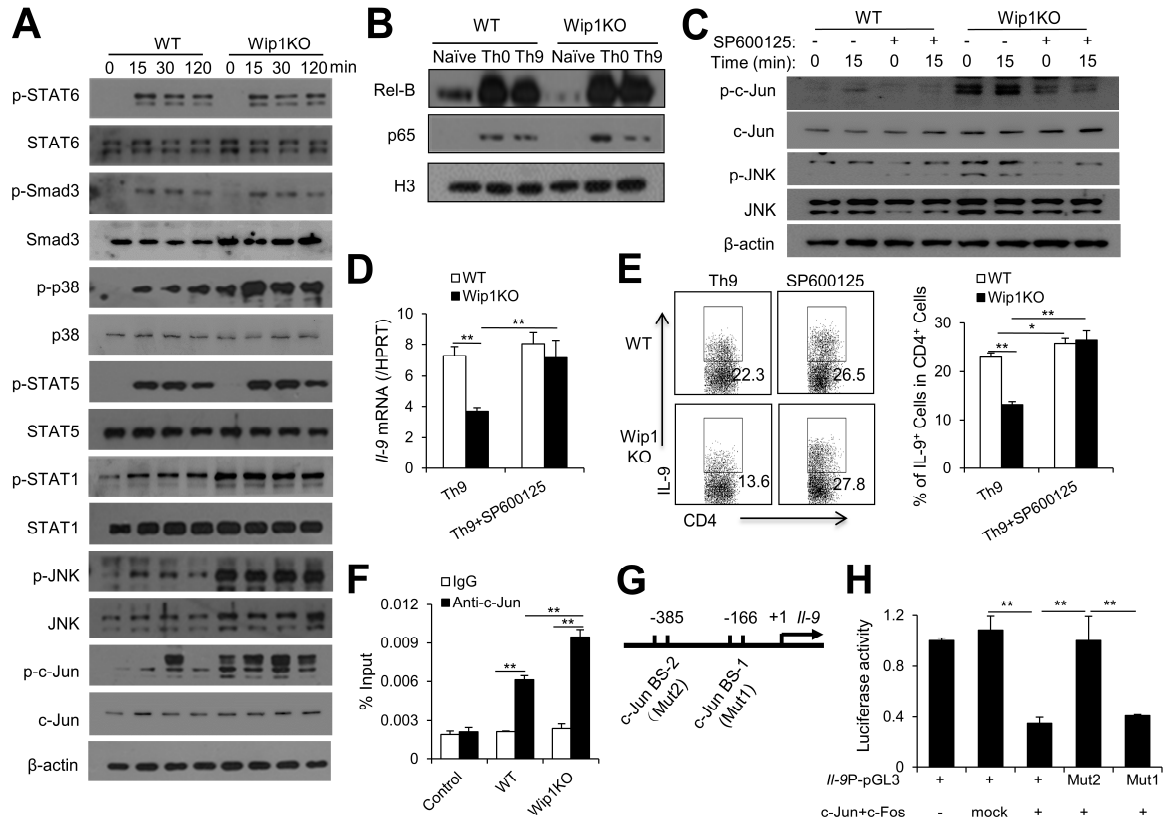
1075 **Table 1. Primers used for genotyping and real-time PCR.**

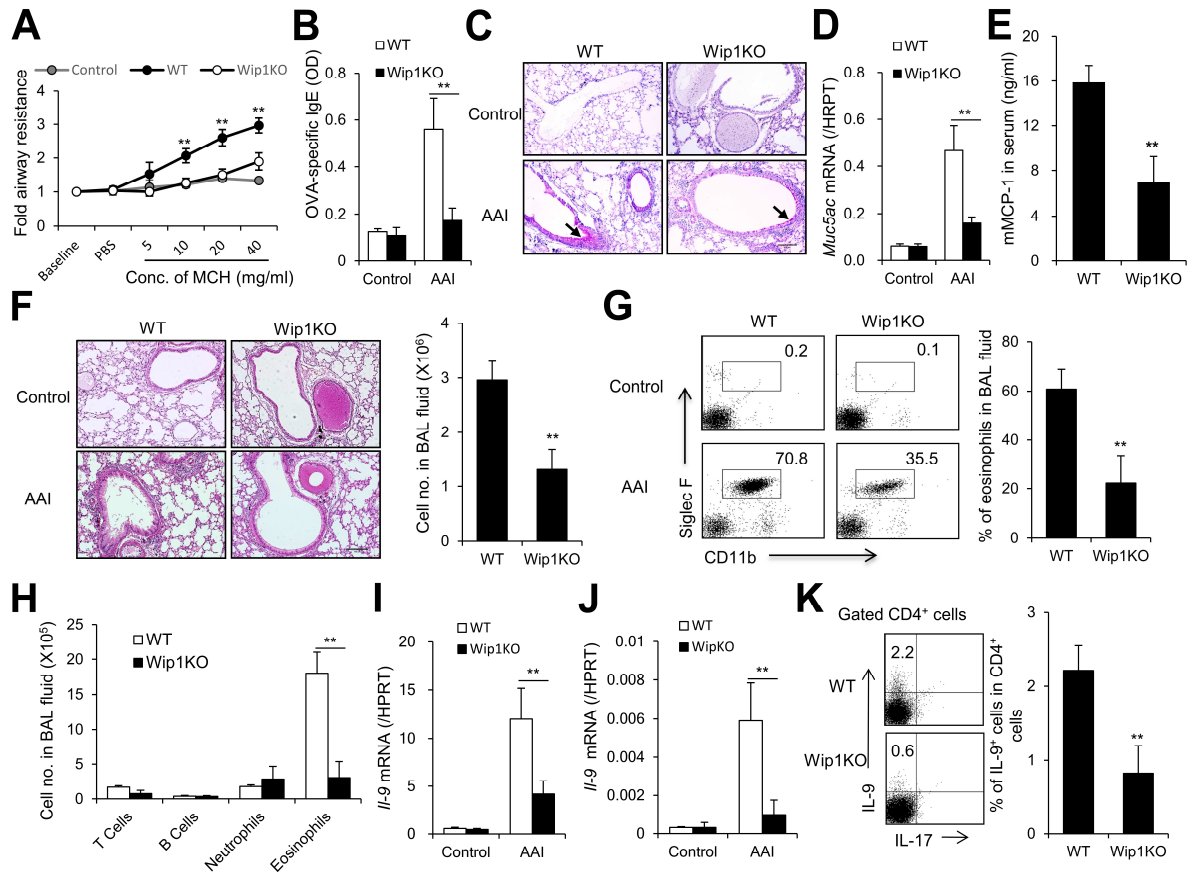
Gene name	Sequence (5'→3')	Usage
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Wip1 Primer2	GGTGACTTGATTGGTGGTGTAGA	
Wip1 Primer3	GCAGGGCTGTTTGTGGTGCT	
Wip1 Primer4	GCATGCTCCAGACTGCCTT	
IL-9	F: CTCTTGCCTGTTTTCCATCG R: CCCAGGAGACTCTTCAGAAATG	RT-PCR
IFN- γ	F: GAACTGGCAAAGGATGGTGA R: TGTGGGTTGTTGACCTCAAAC	RT-PCR
IL-4	F: CGAATGTACCAGGAGCCATATC R: TCTCTGTGGTGTTCCTTCGTTG	RT-PCR
IL-17A	F: CTCAGACTACCTCAACCGTTCC R: ATGTGGTGGTCCAGCTTTCC	RT-PCR
Foxp3	F: ACCATTGGTTTACTCGCATGT R: TCCACTCGCACAAAGCACTT	RT-PCR
T-box21	F: AGCAAGGACGGCGAATGTT R: GGGTGGACATATAAGCGGTTC	RT-PCR
GATA3	F: AGGGACATCCTGCGCGAACTGT R: CATCTTCCGGTTTCGGGTCTGG	RT-PCR
IL-5	F: AGATTCCCATGAGCACAGTG R: TGTCTAGCCCCTGAAAGATTTT	RT-PCR
IL-13	F: GCATGGTATGGAGTGTGGAC R: ATTGGAGATGTTGGTCAGGG	RT-PCR
BCL6	F: CATACAAATGTGATCGCTGCC R: GAGTGTGGGTCTTCAGGTTG	RT-PCR
BATF	F: GCAGTGACTCCAGCTTCAG R: TGTCGGCTTTCTGTGTCTG	RT-PCR
Muc5ac	F: AAAGACATAGTCACTCAGCAA R: CTGGGAAGTTCAGTGTCAAACCA	RT-PCR
Mcpt1	F: TTCCTGATGGCACTTCTCTTG R: CATGTAAGGACGGGAGTGTG	RT-PCR
HPRT	F: AGTACAGCCCCAAAATGGTTAAG R: CTTAGGCTTTGTATTTGGCTTTTC	RT-PCR

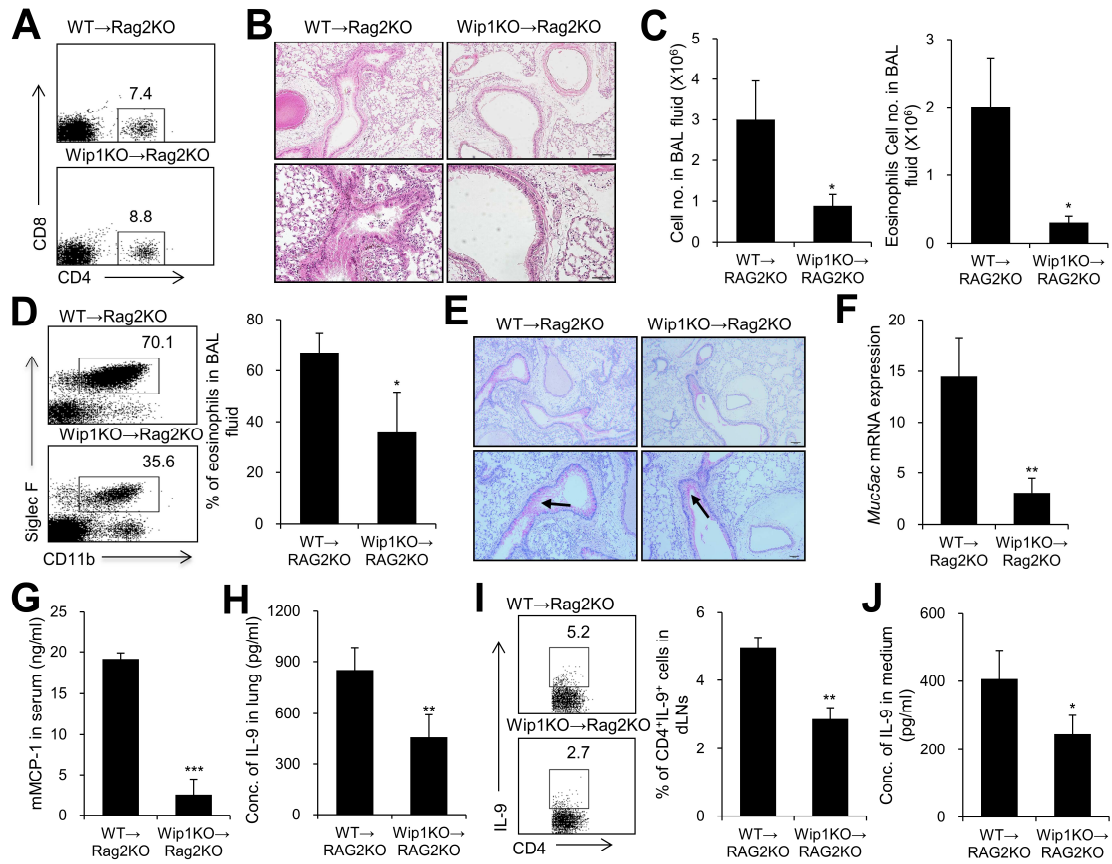
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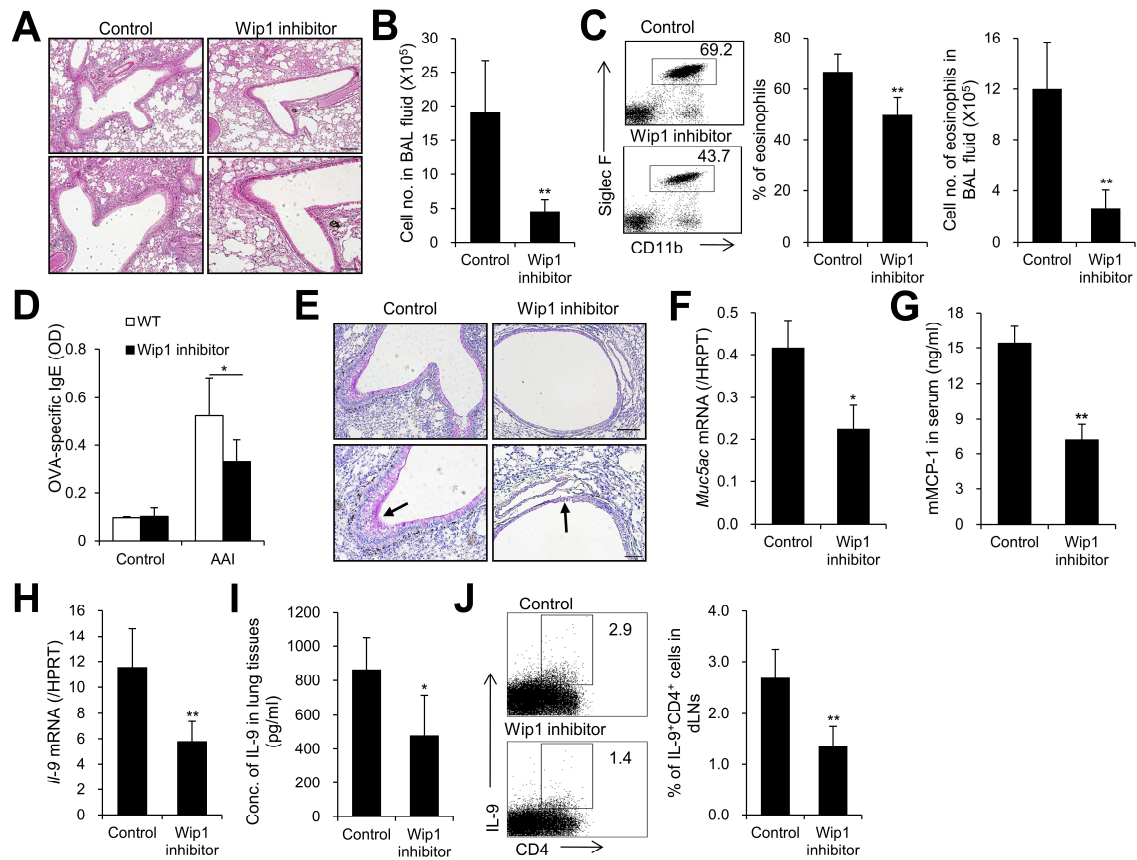












Supplementary figure legends

FIG E1. Th2 cytokines productions of induced Th2 cells of WT and Wip1KO mice *in vitro*.

IL-4 (A), IL-5 (B) and IL-13 (C) levels in supernatants of induced Th2 cells of WT and Wip1KO mice *in vitro*. Data are shown as mean $\pm$ SD.

FIG E2. IL-9 expression under Th2 induction condition.

A, Percentages of IL-9⁺ cells in CD4⁺ T cells of WT and Wip1KO cells under Th2 induction condition. B, IL-9 levels in supernatants of induced Th2 and Th9 cells of WT and Wip1KO mice. C, IL-9 mRNA expression in WT and Wip1KO naïve CD4⁺ T cells under Th0, Th2 and Th9 conditions. Data are shown as mean $\pm$ SD, **P* < .05 ***P* < .01, ****P* < .001.

FIG E3. The mRNA expressions of Th2 cytokines in induced Th9 cells of WT and Wip1KO mice *in vitro*.

A, IL-4, IL-5 and IL-13 mRNA expressions in TGF- β and IL-4 induced Th9 cells and IL-4 induced Th2 cells. B, IL-4, IL-5 and IL-13 mRNA levels in WT and Wip1KO cells during Th9 differentiation *in vitro*. Data are shown as mean $\pm$ SD, ** *P* < .001, *** *P* < .0001.

FIG E4. The effects of Wip1 inhibitor on the induced Th1 cells *in vitro*.

Percentages of IFN- γ ⁺ (A) and TNF- α ⁺ (B) in CD4⁺ T cells of WT cells and cells treated with Wip1 inhibitor under Th1 induction condition. C, T-bet expressions in CD4⁺ T cells of WT cells or cells treated with Wip1 inhibitor under Th1 induction condition. Data are shown as mean $\pm$ SD.

FIG E5. The effects of Wip1 inhibitor on the induced Th2 cells *in vitro*.

A, Percentages of IL-4⁺ in CD4⁺ T cells of WT cells and cells treated with Wip1 inhibitor under Th2 induction condition. B, Gata3 expression in CD4⁺ T cells of WT cells or cells treated with Wip1 inhibitor under Th1 induction condition. Data are shown as mean $\pm$ SD.

FIG E6. The effects of Wip1 inhibitor on the induced Th2 cells *in vitro*.

Percentages of FoxP3⁺ in CD4⁺ T cells of WT cells and cells treated with Wip1 inhibitor under iTreg induction condition. Data are shown as mean $\pm$ SD.

FIG E7. The expressions of Th9 cell related transcription factors in induced Th9 cells of WT and Wip1KO mice.

A, The expressions of PU.1 and IRF4 in CD4⁺ T cells of WT and Wip1KO mice under Th9 induction condition. B, ChIP assays of PU.1, and IRF4 in induced Th9 cells of WT and Wip1KO. ChIP data are shown as control IgG values subtracted from the specific IgG percent input values. C, Gata3 mRNA expressions in induced Th9 and Th2 cells. Batf (D) and Bcl6 (E) mRNA

expressions in induced Th9 cells of WT and Wip1KO mice. Data are shown as mean $\pm$ SD, ** $P < .01$, *** $P < .001$.

FIG E8. The effects of SB203580 and MTA on induced Th9 cells of WT and Wip1KO mice *in vitro*.

A, *Il-9* mRNA expressions in induced Th9 cells of WT and Wip1KO mice treated with different concentrations of SB203580. **B**, Intracellular staining of IL-9 in CD4⁺ T cells under Th9-skewing condition with p38 inhibitor SB203580 (5 μ M). **C**, *Il-9* mRNA expressions in induced Th9 cells of WT and Wip1KO mice treated with different concentrations of MTA. **D**, Intracellular staining of IL-9 in CD4⁺ T cells under Th9-skewing condition with STAT1 inhibitor MTA (10 μ M). Data are shown as mean $\pm$ SD.

FIG E9. The over-expression of c-Jun/c-Fos on *Il-9* expression.

The luciferase activity was assayed as described in the materials and methods. Different doses of c-Jun/c-Fos were transfected. Cells transfected with the *Il-9*-promoter-pGL3 luciferase reporter gene vector alone was used as control as 1. Data are shown as mean $\pm$ SD, * $P < .05$, ** $P < .01$.

FIG E10. The levels of OVA-specific IgGs in serum of WT and Wip1KO mice in an OVA-induced allergic airway inflammation model.

OVA-specific IgG levels (**A**), OVA-specific IgG1 levels (**B**) and OVA-specific IgG2b levels (**C**) of WT and Wip1KO mice in OVA-induced airway inflammation. Data are shown as mean $\pm$ SD, ** $P < .01$.

FIG E11. *Mcpt1* mRNA expression in lung tissues of WT and Wip1KO mice in an OVA-induced allergic airway inflammation model.

Data are shown as mean $\pm$ SD, ** $P < .01$.

FIG E12. The mRNA expressions of Th1, Th2 and Th17 cytokines in BAL cells of WT and Wip1KO mice in an OVA-induced allergic airway inflammation model.

Il-4 (**A**), *Il-5* (**B**) and *Il-13* (**C**), *Ifng* (**D**) and *Il-17* (**E**) mRNA expression in BAL cells of WT and Wip1KO mice in OVA-induced allergic airway inflammation model. Data are shown as mean $\pm$ SD.

FIG E13. *Mcpt1* mRNA expression in lung tissues of Rag2KO mice received WT and Wip1KO CD4⁺ T cells in an OVA-induced allergic airway inflammation model.

Data are shown as mean $\pm$ SD, ** $P < .01$.

FIG E14. The mRNA expressions of Th2 cytokines in BAL cells of RAG2KO mice received WT and Wip1KO CD4⁺ T cells in OVA-induced allergic airway inflammation model.

Il-4 (A), *Il-5* (B) and *Il-13* (C) mRNA expressions in BAL cells isolated from WT or Wip1KO CD4⁺ T cells-transferred RAG2KO mice in OVA-induced allergic airway inflammation model. Data are shown as mean $\pm$ SD.

FIG E15. *Mcpt1* mRNA expression in lung tissues of mice treated with buffer and Wip1 inhibitor in an allergic airway inflammation model.

Data are shown as mean $\pm$ SD, ** $P < .01$.

FIG E16. The effects of Wip1 inhibitor on the productions of Th2 cytokines and TNF- α in an OVA-induced allergic airway inflammation model.

A, *Il-4*, *Il-5*, *Il-13*, *Ifng* and *Il-17* mRNA expressions in lung tissues of WT mice and mice treated with Wip1 inhibitor in OVA-induced allergic airway inflammation. B, IL-4, IL-5 and IL-13 levels in supernatants of OVA-stimulated dLNs cells of WT mice and mice treated with Wip1 inhibitor in OVA-induced allergic airway inflammation. C, TNF- α levels in BAL fluid and supernatants of splenocytes stimulated with OVA of WT mice and mice treated with Wip1 inhibitor in OVA-induced allergic airway inflammation. Data are shown as mean $\pm$ SD.

FIG E17. FoxP3 and T-bet expressions in mice treated with Wip1 inhibitor during OVA-induced allergic airway inflammation.

Percentages of FoxP3⁺ cells (A) and T-bet⁺ cells (B) in of BAL CD4⁺ cells of WT mice and mice treated with Wip1 inhibitor. *Foxp3* mRNA (C) and *Tbx21* mRNA (D) expressions in lung tissues and dLNs cells of WT mice and mice treated with Wip1 inhibitor. Data are shown as mean $\pm$ SD.

