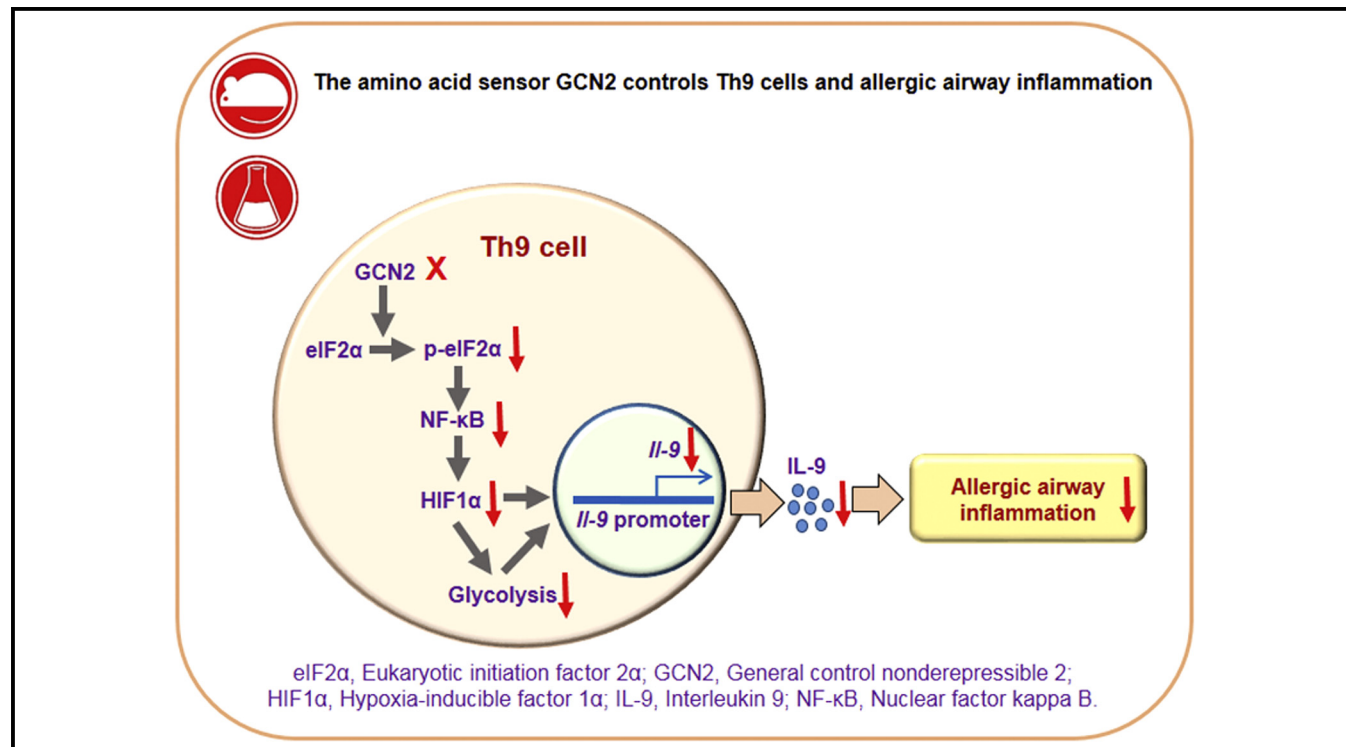


The amino acid sensor general control nonderepressible 2 (GCN2) controls T_H9 cells and allergic airway inflammation



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GRAPHICAL ABSTRACT



Background: T_H9 cells have emerged as important mediators of allergic airway inflammation. There is evidence that general control nonderepressible 2 (GCN2) affects the immune response under some stress conditions. However, whether GCN2 regulates CD4⁺ T-cell differentiation during allergic inflammation remains unknown.

Objective: We sought to clarify the regulatory roles of GCN2 in CD4⁺ T-cell subset differentiation and its significance in patients with allergic airway inflammation.

Methods: The effects of GCN2 in differentiation of T_H cell subsets were detected by using the *in vitro* induction system. GCN2 knockout mice, ovalbumin-induced allergic airway inflammation, and adoptive transfer mouse models were used to determine the significance of GCN2 in T_H9 differentiation and allergic airway inflammation *in vivo*. RNA sequencing, real-time PCR, Western blotting, and other molecular approaches were used to identify the molecular mechanisms relevant to regulation of GCN2 in T_H9 cell differentiation.

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Results: GCN2 deficiency significantly inhibited differentiation of T_H9 cells but not T_H1, T_H2, and regulatory T cells. GCN2 knockout mice and recombination-activating gene 2 knockout (Rag2KO) mice that received adoptively transferred GCN2-deficient CD4⁺ T cells exhibited reduced T_H9 differentiation and less severe allergic airway inflammation. Furthermore, the isolated GCN2-deficient T_H9 cells also mediated less severe allergic airway inflammation on adoptive transfer. Mechanistically, GCN2 deficiency inhibits T_H9 cell differentiation through a hypoxia-inducible factor 1 α -dependent glycolytic pathway.

Conclusion: Our results reveal a novel role of GCN2 in T_H9 cell differentiation. Our findings indicate that new strategies to inhibit GCN2 activity might provide novel approaches to attenuate allergic airway inflammation. (J Allergy Clin Immunol 2019;144:1091-105.)

Key words: GCN2, T_H9 cell, allergic airway inflammation, metabolism

Allergic asthma is a chronic multicellular inflammatory disease of the airways that is characterized by infiltration of eosinophils, basophils, mast cells, and CD4⁺ T_H cells into the airway submucosa.¹ The pathophysiology of allergic asthma includes mucus hypersecretion, airway wall remodeling, and airway hyperresponsiveness.^{1,2} Because T_H2 cell-secreted cytokines (IL-4, IL-5, and IL-13) play critical roles in the pathogenesis of allergic inflammation,³ allergic asthma has been regarded as a T_H2 cell-associated disease for a long time. However, recent evidence from patients and allergic asthma models suggests that T_H9 cells and their secreted IL-9 were also involved in the pathogenesis of allergic asthma.⁴⁻⁷ For example, the number of circulating T cells that could produce IL-9 in response to specific antigens was markedly increased in allergic patients,^{7,8} and several genes associated with T_H9 cell differentiation have been linked to allergic diseases in human subjects.^{6,9}

Using IL-9 fate reporter mice, Wilhelm et al¹⁰ nicely demonstrated that T_H9 cells were the main source of IL-9 in the setting of allergic airway inflammation. The proallergic cytokines that promote T_H9 cells and IL-9 production, such as IL-25 and thymic stromal lymphopoietin, also significantly aggravated allergic airway inflammation.^{11,12} By contrast, inhibiting T_H9 cell differentiation or IL-9 production significantly alleviated allergic airway inflammation.¹³⁻¹⁵ Therefore intervening in T_H9 cells and IL-9 might be a potential therapeutic approach for allergic asthma treatment. However, it is also reported that IL-9 might not be significantly involved in patients with allergic airway inflammation.¹⁶ This inconsistency requires future study.

General control nonderepressible 2 (GCN2), a eukaryotic initiation factor 2 α (eIF2 α) kinase, is activated by uncharged transfer RNA accumulation under conditions of amino acid starvation.^{17,18} GCN2 is closely involved in many biological processes, including memory formation, cell apoptosis, and metabolism. Interestingly, GCN2 exerts a biphasic regulation of inflammation in the immune response. Myeloid deletion of GCN2 in lupus-prone mice resulted in increased immune cell activation, humoral autoimmunity, renal pathology, and mortality.¹⁹ In contrast, monocytic lineage-specific GCN2 knockout (GCN2KO) mice showed reduced inflammatory responses and mortality after a lethal dose of LPS treatment.²⁰ GCN2 deficiency decreased infiltration of regulatory T (Treg)

Abbreviations used

BALF:	Bronchoalveolar lavage fluid
BATF:	Basic leucine zipper transcription factor
DEG:	Differentially expressed gene
2-DG:	2-Deoxy-d-glucose
dLN:	Draining lymph node
eIF2 α :	Eukaryotic initiation factor 2 α
Foxp3:	Forkhead box P3
GCN2:	General control nonderepressible 2
GCN2KO:	GCN2 knockout
H&E:	Hematoxylin and eosin
HIF:	Hypoxia-inducible factor
IRF4:	Interferon regulatory factor 4
iTreg:	Induced regulatory T cell
KEGG:	Kyoto Encyclopedia of Genes and Genomes
MCPT-1:	Mast cell protease 1
NF- κ B:	Nuclear factor κ B
OVA:	Ovalbumin
PAS:	Periodic acid-Schiff
PMA:	Phorbol 12-myristate 13-acetate
Rag2KO:	Recombination-activating gene 2 knockout
RNA-Seq:	RNA sequencing
STAT6:	Signal transducer and activator of transcription 6
Treg:	Regulatory T
WT:	Wild-type

cells into the inflamed central nervous system in patients with experimental autoimmune encephalomyelitis.²¹ It has been reported that activation of GCN2 by halofuginone inhibits T_H17 cell differentiation.²² Deletion of GCN2 specifically in CD11c⁺ cells or intestinal epithelial cells increased intestinal inflammation and the T_H17 response.²³ However, whether the integrated stress response kinase GCN2 directly affects other CD4⁺ T_H subset differentiation has not been studied.

In this study we investigated the effect of GCN2 on CD4⁺ T-cell function differentiation using GCN2KO mice and wild-type (WT) littermate mice. Our results showed that GCN2 deficiency significantly inhibited T_H9 cell differentiation in an intrinsic manner but had no obvious effect on differentiation of T_H1, T_H2, and induced regulatory T (iTreg) cells. Using a mouse model of allergic airway inflammation induced by ovalbumin (OVA) sensitization, we found that deletion of GCN2 or adoptive transfer of GCN2-deficient naive CD4⁺ T cells attenuated allergic airway inflammation, which was associated with reduced T_H9 cell differentiation and IL-9 production. Furthermore, adoptive transfer of isolated GCN2-deficient T_H9 cells also significantly reduced allergic airway inflammation. Taken together, these data indicate that GCN2 might be a potential intervention target to treat allergic airway inflammation.

METHODS

Mice

GCN2KO mice were obtained from the Jackson Laboratory (Bar Harbor, Me) and then crossed with the C57BL/6J (B6) strain (obtained from Huafukang Bioscience, Beijing, China).²⁴ B6 and recombination-activating gene 2 knockout (Rag2KO) mice were purchased from Beijing University Experimental Animal Center (Beijing, China) and Huafukang Bioscience (Beijing, China), respectively. All mice were bred and maintained under specific pathogen-free conditions in the animal facility of the Institute of Zoology, Chinese Academy of Science. Sex-matched littermate WT and

GCN2KO mice 8 to 12 weeks of age 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 cells *in vitro*

Naive CD4⁺ T cells (CD4⁺CD44⁺CD62L⁺) were sorted from spleens of WT or GCN2KO mice by using the MoFlo XDP (Beckman Coulter, Fullerton, Calif) or magnetic cell sorting (STEMCELL Technologies, Vancouver, British Columbia, Canada). Sorted naive CD4⁺ T cells were cultured with RPMI 1640 supplemented with 10% FCS (Gibco, Carlsbad, Calif), 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.05 mol/L 2-mercaptoethanol. Sorted naive CD4⁺ T cells were activated with plate-bound anti-CD3 (2 µg/mL; 145-2C11; BD Biosciences, San Jose, Calif) and soluble anti-CD28 (2 µg/mL; 37.51; BD Biosciences).²⁵ Meanwhile, these cells were cultured under different cytokine environments for T_H subset differentiation.^{13,26} T_H9 induction conditions were as follows: 20 ng/mL IL-4 (R&D Systems, Minneapolis, Minn), 2 ng/mL TGF-β (R&D Systems), and 10 µg/mL anti-IFN-γ (XMG1.2; eBioscience, San Diego, Calif). T_H1 induction conditions were as follows: 10 ng/mL IL-12 (R&D Systems), 100 U/mL IL-2 (PeproTech, Rocky Hill, NJ), and 10 µg/mL anti-IL-4 (11B11; eBioscience). T_H2 induction conditions were as follows: 20 ng/mL IL-4 (R&D Systems) and 10 µg/mL anti-IFN-γ (XMG1.2; eBioscience). T_H17 induction conditions were as follows: 1 ng/mL TGF-β (R&D Systems), 50 ng/mL IL-6 (R&D Systems), 10 µg/mL anti-IFN-γ (XMG1.2; eBioscience), and 10 µg/mL anti-IL-4 (11B11; eBioscience). iTreg cell induction conditions were as follows: 1 ng/mL TGF-β (R&D Systems) and 100 U/mL IL-2 (PeproTech). After 3 days or the indicated time points of differentiation, cell differentiation was assessed by using intracellular staining and real-time PCR.

Flow cytometry

After 3 to 5 days of culture, cells were stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) and 500 ng/mL ionomycin (Sigma-Aldrich, St Louis, Mo) for 5 hours at 37°C, and then GolgiStop (BD Pharmingen) was added to the cells for the last 3 hours of stimulation. Intracellular cytokine staining was carried out with Fixation/Permeabilization buffer solution, according to the manufacturer's protocol (BD Biosciences). Fluorochrome-conjugated anti-mouse IL-9 (RM9A4; BioLegend, San Diego, Calif), anti-mouse IL-13 (eBio13A; eBioscience), anti-mouse IL-17A (TC11; BioLegend), anti-mouse IFN-γ (XMG1.2; BioLegend), anti-mouse T-bet (4B10; BioLegend), and anti-mouse forkhead box P3 (Foxp3; NRRF-30; eBioscience) were used for staining. Foxp3 T-bet staining was carried out with Fixation/Permeabilization buffer solution, according to the manufacturer's protocol (eBioscience). Stained cells were analyzed on the Epics XL (Beckman Coulter), and obtained data were analyzed with FCS Express 3 software (De Novo Software, Glendale, Calif).

Real-time PCR

Total RNA was extracted from cultured cells with a total RNA kit (Promega, Madison, Wis), and cDNA was synthesized with the cDNA Reverse Transcription Kit (Takara Bio, Shiga, Japan).^{27,28} Quantitative real-time PCR was conducted on the CFX96 Real-Time System (Bio-Rad Laboratories, Hercules, Calif) by using Power SYBR PCR Master Mix (Applied Biosystems, Foster City, Calif). Every sample was run in triplicates. The primers used are listed in Table I. Real-time PCR data were analyzed by using the comparative cycle threshold method and were normalized by using the housekeeping gene *Hprt*.

ELISA

Homogenates of lung tissues was prepared with RIPA buffer.¹³ IL-9 levels in cell-culture supernatants and homogenates of lung tissue were measured with ELISA kit (eBioscience and BioLegend). Mouse mast cell protease 1 (MCPT-1) was detected by using mouse MCPT-1 (eBioscience) ELISA kits,

TABLE I. Primers used for genotyping and real-time PCR

Gene name	Sequence (5' → 3')	Use
GCN2 primer 1	TCTCCCAGCGGAATCCGCACATCG	Genotyping
GCN2 primer 2	ATCCAGGCGTTGTAGTAGCGCACA	
GCN2 primer 3	TGCCACTGTGCAAGTCTGAAGCAGG	RT-PCR
<i>Ii9</i>	F: CTCTTGCTGTTTTCATCG R: CCCAGGAGACTCTTCAGAAATG	
<i>Ifng</i>	F: GAACTGGCAAAAGGATGGTGA R: TGTGGGTTGTGACCTCAAAC	RT-PCR
<i>Il4</i>	F: CGAATGTACAGGAGCCATATC R: TCTCTGTGTCTTCTTCGTTG	
<i>Il17a</i>	F: CTCAGACTACCTCAACCGTTCC R: ATGTGGTGGTCCAGCTTTCC	RT-PCR
<i>Foxp3</i>	F: ACCATTGGTTTACTCGCATGT R: TCCACTCGCACAAAGCACTT	
T-box 21	F: AGCAAGGACGGCGAATGTT R: GGTGGACATAAAGCGGTTT	RT-PCR
<i>Gata3</i>	F: AGGGACATCTGCGCAACTGT R: CATCTTCCGGTTTCGGGTCTGG	
<i>Rorc</i>	F: TTTCTGAGGATGAGATTGCC R: TTGTCGATGAGTCTTGACAGAG	RT-PCR
<i>Spi1</i>	F: GTCTGATGGAGAAGCTGATGG R: AGATGCTGTCTTCTGCTGTCG	
<i>Irf4</i>	F: ACCAGTCACACCCAGAAATC R: GGGCACAAGCATAAAAGGTTT	RT-PCR
<i>Hif1a</i>	F: GAACATCAAGTCAGCAACGTG R: TTTGACGGATGAGGAATGGG	
<i>Pfkl</i>	F: GGAAAGCCTATCTCATCCAGC R: CCATACCCTCTTGCTACTCAG	RT-PCR
<i>Eno1</i>	F: CGCCATGTCTATTCTCAGGATC R: AGTTCTAGGGCCTCGTAGATG	
<i>Hk2</i>	F: TCAAAGAGACAAGGGCGAG R: AGGAAGCGGACATACAATC	RT-PCR
<i>Gcn2</i>	F: TCTCCCAGTCCCTTTTACCTG R: TGTCACTGAAGGCTCAATCTC	
<i>Hprt</i>	F: AGTACAGCCCCAAAATGGTTAAG R: CTTAGGCTTTGTATTTGGCTTTTC	RT-PCR

F, Forward; R, reverse.

and mouse OVA-specific IgG_{2b} and IgG₁ in sera were detected by using a quantitative ELISA kit of mouse anti-OVA IgG_{2b} (Chondrex, Redmond, Wash) and mouse anti-OVA IgG₁ (Chondrex). All these kits were used according to the manufacturer's instructions.

Lactate measurement

After sorted WT and GCN2KO CD4⁺ naive T cells were cultured under T_H9-skewing conditions for 3 days, supernatants were collected, and lactate levels were measured by using the Lactate Assay Kit and spectrophotometry (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the manufacturer's protocol. Induction of allergic airway inflammation.

A mouse allergic airway inflammation model was induced, as previously described.^{13,15,26} Mice 8 to 10 weeks of age were intraperitoneally injected with 10 µg of OVA (Sigma-Aldrich) mixed with 1 mg of aluminum hydroxide (Sigma-Aldrich) on days 0 and 14. From day 21, mice were sealed in cages (exposure chamber volume, about 7.4 L) and whole-body challenged with aerosolized OVA (1% in PBS) for 30 minutes per day for 5 consecutive days with ultrasonic nebulization (NB-150U; Omron, Shenzhen, China). The nebulizer could atomize OVA into tiny fog particles (1–5 µm), and the average nebulizing speed was 1.5 mL/min. Mice were killed 48 hours after the last challenge. The mouse allergic airway inflammation model with transferred cells was as previously described.^{13,29}

Sorted CD4⁺ T cells (1 × 10⁷) of WT and GCN2 KO mice were intravenously injected to recombination-activating gene 2 knockout (Rag2KO) mice, respectively, by 1 day before OVA sensitization. These

mice then underwent OVA-induced allergic airway inflammation, as described above. OT-II transgenic mice, which express the mouse α -chain and β -chain T-cell receptor that pairs with the CD4 coreceptor and is specific for chicken OVA₃₂₃₋₃₃₉ peptide in the context of I-A^b, were used to prove specific immune responses. Sorted naive CD4⁺ T cells from WT-OT-II and GCN2KO-OT-II mice were cultured under T_H9-skewing conditions for 3 days with OVA₃₂₃₋₃₃₉ peptide, and then these cells were collected (2×10^6) and adoptively transferred into naive WT B6 recipient mice, respectively. These recipient mice were then exposed to aerosolized OVA for 30 min/d for 5 days, as described above.^{26,30}

The trachea was cannulated, and lungs were lavaged with 3 mL of PBS. Cell numbers in bronchoalveolar lavage fluid (BALF) were counted with a hemocytometer. Cells in BALF were analyzed by using flow cytometry, with antibodies against 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. Peripheral immune responses in sensitized and challenged mice were assessed by means of culture of draining lymph nodes (dLNs) with PMA and ionomycin. Lung tissue samples were prepared and embedded in paraffin and sectioned and then stained with hematoxylin and eosin (H&E) and periodic acid–Schiff (PAS) for evaluation of the infiltration of inflammatory cells and mucin production in the trachea.³¹

Histopathologic analysis

Mouse lung tissue samples were prepared, embedded in paraffin, sectioned, and stained with H&E, PAS, and toluidine blue, respectively.³² To quantify tissue histopathology, we adopted a semiquantitative score system (0–4, with 0 denoting no inflammation) to evaluate pulmonary inflammation.^{15,33} Slides were examined in blind fashion by 2 experienced observers with a microscope, and the following indicators were mainly considered: peribronchial and perivascular cellular infiltration, as well as fibrosis. Three randomly chosen areas in each slide were scored, and each group contained 3 to 5 samples. For PAS staining analysis, percentages of areas of PAS⁺ cells were assessed by using the IHC Toolbox function in ImageJ software (National Institutes of Health, Bethesda, Md).

Immunoblotting

Naive CD4⁺ T cells were cultured in RPMI 1640 medium with 10% FBS. After stimulation, cells were washed once in cold PBS and lysed in RIPA buffer (50 mmol/L Tris–HCl [pH 7.4], 1% NP-40, 0.25% Na-deoxycholate, 150 mmol/L NaCl, and 1 mmol/L EDTA [pH 7.4]) with protease and phosphatase inhibitor cocktails (Roche) for 10 minutes on ice.¹³ Protein concentrations were determined by using the bicinchoninic acid assay. Lysates were mixed with 5× SDS-PAGE loading buffer and boiled at 100°C for 5 minutes. Protein samples were size fractionated on 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Millipore, Temecula, Calif).^{34,35} After an hour of blocking, membranes were incubated with primary antibodies overnight at 4°C. The appropriate horseradish peroxidase–conjugated secondary antibody was then added and detected through chemiluminescence (Millipore). Actin was used as a protein-loading control. Phosphorylated signal transducer and activator of transcription 6 (STAT6; Tyr641), STAT6, phosphorylated Smad2 (Ser423/425), Smad2, phosphorylated nuclear factor κ B (NF- κ B) p65, NF- κ B p65 (E498), phosphorylated eIF2 α (Ser51), eIF2 α , PU.1 (9G7), interferon regulatory factor 4 (IRF4; P173), basic leucine zipper transcription factor (BATF; D7C5), hypoxia-inducible factor (HIF) 1 α , β -actin, and histone H3 antibodies were all purchased from Cell Signaling Technology (Danvers, Mass).

RNA sequencing analysis

We sorted the *in vitro*-induced T_H9 cells of naive CD4⁺ T cells for transcriptomic analysis. Specifically, a total amount of 1.5 μ g of RNA was extracted by using TRIzol Reagent (Invitrogen, Carlsbad, Calif), and RNA purity was checked by using the NanoDrop 1000 (Agilent Technologies, Santa

Clara, Calif). Sequencing libraries were generated by using the NEBNext UltraTM RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, Mass), according to the manufacturer's recommendations, and index codes were added to attribute sequences to each sample. Briefly, mRNA was purified from total RNA by using poly-T oligo-attached magnetic beads. Fragmentation was carried out with divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5×). First-strand cDNA was synthesized by using random hexamer primer and M-MuLV Reverse Transcriptase (RNaseH-). Second-strand cDNA synthesis was subsequently performed by using DNA polymerase I and RNase H. Remaining overhangs were converted into blunt ends through exonuclease/polymerase activities.

After adenylation of 3' ends of DNA fragments, NEBNext Adaptors with hairpin loop structure were ligated to prepare for hybridization. Library fragments were purified with the AMPure XP system (Beckman Coulter) to select cDNA fragments with the right length. Then 3 μ L of USER Enzyme (New England Biolabs) was used with size-selected, adaptor-ligated cDNA at 37°C for 15 minutes, followed by 5 minutes at 95°C before PCR. Then PCR was performed with Phusion High-Fidelity DNA Polymerase, universal PCR primers and Index (X) Primer. Lastly, products were purified (AMPure XP System), and library quality was assessed on the Agilent Bioanalyzer 2100 system.

The normalized expression value for each transcript was calculated as fragments per kilobase million to characterize the gene expression profile of GCN2KO cells. The R package DESeq v1.32.052 was used to compare gene expression profiles of T_H9 CD4⁺ naive T cells in GCN2KO and WT mice. The criteria for calculating differentially expressed genes (DEGs) were based on fragments per kilobase million values and reported as fold changes and *P* values. We have shown that transcripts with a fold change of greater than |1.5| and *P* values of less than .05 were assigned as statistically significant differential expression.

Pathway and network analysis

We compared all the statistically significant DEGs produced by analysis of T_H9, T_H0, and naive CD4⁺ T cells between the transcriptomes of GCN2KO and WT mice, respectively, and then performed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis on their different DEGs clusters. Terms and route categories with *P* values of .05 or less in the hypergeometric test were selected for further analysis. Signal pathway analysis was performed with the KOBAS 3.0 online tool 1 and Java GSEA2. Visualization was performed with the R packages ggplot v2 2.2.1 and pheatmap v1.0.8.

The transcription factor annotations of *Mus musculus* were downloaded from AnimalTFDB3. We used the Network Analyst 4 online tool to generate a network of transcription factors and their interactions by inputting transcription factor genes involved in significant DEGs in T_H9 conditions. For the functional gene network, we retained the gene set of fold change of greater than |1.5| in the network, and the results were visualized by using Cytoscape.

Statistical analysis

All data are presented as means $\pm$ SDs. The unpaired 2-tailed Student *t* test was used for data from 2 groups. Data from more than 2 groups were subjected to 1-way ANOVA with SAS (version 9.2; SAS Institute, Cary, NC). Comparisons were considered statistically significant when the *P* value was less than .05.

RESULTS

GCN2 is dispensable for differentiation of T_H1, T_H2, and Treg cells

We first confirmed deletion of GCN2 at both the mRNA and protein levels in CD4⁺ T cells of GCN2KO mice (see Fig E1 in this article's Online Repository at www.jacionline.org). Then we measured percentages of CD4⁺ and CD8⁺ T cells in spleens

and peripheral lymph nodes and found there were no differences in the percentages of peripheral T cells between WT and GCN2KO mice in physiologic situations (see Fig E2, A and B, in this article's Online Repository at www.jacionline.org). Meanwhile, GCN2 deficiency had no obvious effect on percentages of naive T cells in spleens (see Fig E2, C).

To determine whether GCN2 affected CD4⁺ T-cell proliferation and activation, which are important for T_H cell differentiation, we performed carboxyfluorescein succinimidyl ester dilution assays and measured the expression of activation markers of CD4⁺ T cells. The results showed that sorted naive CD4⁺ T cells from GCN2-deficient mice possess identical proliferation capability as those of WT mice after activation by anti-CD3 and anti-CD28 mAbs *in vitro* (see Fig E3, A, in this article's Online Repository at www.jacionline.org). Similarly, GCN2-deficient CD4⁺ T cells also showed equivalent expression of activation makers, including the early activation marker CD69 and the late activation marker CD25 (see Fig E3, B and C). Therefore GCN2 is dispensable for the proliferation and activation of the sorted naive CD4⁺ T cells *in vitro*.

Next, we investigated the effect of GCN2 on differentiation of CD4⁺ T cells under diverse *in vitro* T-cell function differentiation conditions. First, real-time PCR results and flow cytometric assays showed that expression of *Ifng* mRNA and percentages of CD4⁺IFN- γ ⁺ T_H1 cells were similar in sorted WT and GCN2-deficient naive CD4⁺ T cells under T_H1-skewing condition (see Fig E4, A, in this article's Online Repository at www.jacionline.org). Second, GCN2 deficiency did not affect expression of *Il4* and percentages of CD4⁺IL-4⁺ T_H2 cells in sorted naive CD4⁺ T cells under T_H2-skewing conditions (see Fig E4, B). Third, GCN2 deficiency slightly but significantly decreased mRNA levels of *Il17a* and percentages of CD4⁺IL-17A⁺ T_H17 cells in sorted naive CD4⁺ T cells under T_H17-skewing conditions (see Fig E4, C). Because Foxp3 is a master transcription factor and the key maker of Treg cells,³⁶ we measured Foxp3 levels in naive CD4⁺ T cells under Treg cell-skewing conditions and found that neither the expression of Foxp3 nor the percentage of CD4⁺Foxp3⁺ iTreg cells was affected by GCN2 deficiency (see Fig E4, D).

To determine whether GCN2 affects expression of the respective master transcription factors in T_H1, T_H2, and T_H17 cells, respectively, during differentiation, we measured mRNA levels of T-bet, GATA-3, and retinoic acid-related orphan receptor γ t in sorted naive CD4⁺ T cells under respective differentiation conditions by using real-time PCR. Results showed that GCN2 deficiency did not detectably influence mRNA expression of the master transcription factors in T_H1, T_H2, and T_H17 cells, respectively (see Fig E5 in this article's Online Repository at www.jacionline.org). Therefore GCN2 deficiency in CD4⁺ T cells does not intrinsically affect differentiation of T_H1, T_H2, and iTreg cells under their respective induction conditions while slightly decreasing T_H17 cell differentiation under T_H17-skewing conditions *in vitro*.

GCN2 deficiency attenuates T_H9 cell differentiation *in vitro*

Sorted WT and GCN2-deficient naive CD4⁺ T cells were cultured under T_H9-skewing conditions to investigate the effect of GCN2 on IL-9 expression in T_H9 cells. Compared with WT naive CD4⁺ T cells, GCN2-deficient naive CD4⁺ T cells showed

significantly lower percentages of CD4⁺IL-9⁺ T_H9 cells ($P < .01$; Fig 1, A). *Il9* mRNA levels were significantly lower in GCN2-deficient CD4⁺ T cells than in WT CD4⁺ T cells at different time points under T_H9 induction conditions ($P < .01$; Fig 1, B). Consistently, IL-9 protein levels in culture medium of GCN2-deficient CD4⁺ T cells under T_H9 induction condition were significantly lower than those in WT CD4⁺ T cells, as detected by means of ELISA ($P < .01$; Fig 1, C). Next, we sorted naive CD4⁺ T cells from WT-OT-II and GCN2KO-OT-II mice and then stimulated these cells with OVA₃₂₃₋₃₃₉ peptide under T_H9 induction conditions. Sorted naive GCN2KO-OT-II CD4⁺ T cells showed remarkably lower percentages of CD4⁺IL-9⁺ T_H9 cells under T_H9-skewing conditions than sorted WT-OT-II CD4⁺ T cells after antigen stimulation under T_H9 induction conditions ($P < .01$; Fig 1, D).

It has been reported that GCN2 is involved in cell cycle regulation.³⁷ Therefore we performed the carboxyfluorescein succinimidyl ester assay to further determine whether the effect of GCN2 deficiency on T_H9 cell differentiation was simply due to the attenuated proliferation ability of CD4⁺ T cells under T_H9 induction conditions. After culture for 3 days, there was no obvious difference in proliferation ability between WT and GCN2-deficient CD4⁺ T cells. However, percentages of CD4⁺IL-9⁺ T_H9 cells from GCN2-deficient CD4⁺ T cells were still significantly lower than those from WT CD4⁺ T cells ($P < .01$; Fig 1, E).

It is well known that T_H cells possess plasticity and flexibility and can differentiate into another subset of T_H cells under specific cytokine microenvironments. Therefore we measured the expression of master transcription factors and specific markers of different subsets of T_H cells to determine whether GCN2 deficiency could promote naive CD4⁺ T cells to differentiate into other subtypes of T_H cells under T_H9 induction conditions. We observed that percentages of Foxp3⁺ T cells and T-bet⁺ T cells were very low in naive CD4⁺ T cells cultured under T_H9-skewing conditions and that there was no significant difference between WT and GCN2-deficient cells (Fig 1, F and G). In addition, there were very few IFN- γ ⁺, IL-4⁺, and IL-17⁺ T cells among WT and GCN2-deficient naive CD4⁺ T cells under T_H9 induction conditions (see Fig E6 in this article's Online Repository at www.jacionline.org). Together, these data suggest that the attenuated T_H9 cell differentiation in GCN2-deficient naive CD4⁺ T cells was not caused by skewing differentiation of naive CD4⁺ T cells into T_H1, T_H2, T_H17, and iTreg cells under T_H9 induction conditions.

GCN2 deficiency attenuates T_H9 cell differentiation *in vivo* and allergic airway inflammation

The OVA-induced allergic airway inflammation mouse model is a kind of acute allergen challenge model of allergic asthma and has been widely used to elucidate the functions of T_H9 cells and IL-9.^{7,14,15} To investigate the effects of GCN2 on T_H9 cells *in vivo*, we challenged WT and GCN2KO mice with OVA, which significantly increased serum levels of IgE, IgG₁, and IgG_{2b} in WT and GCN2KO mice (Fig 2, A, and see Fig E7, A, in this article's Online Repository at www.jacionline.org). However, GCN2KO mice exhibited significantly lower serum levels of OVA-specific IgG₁ and IgE than WT mice ($P < .01$; Fig 2, A, and see Fig E7, A). Meanwhile, there was no significant difference in T_H1-dependent OVA-specific IgG_{2b} levels between WT and

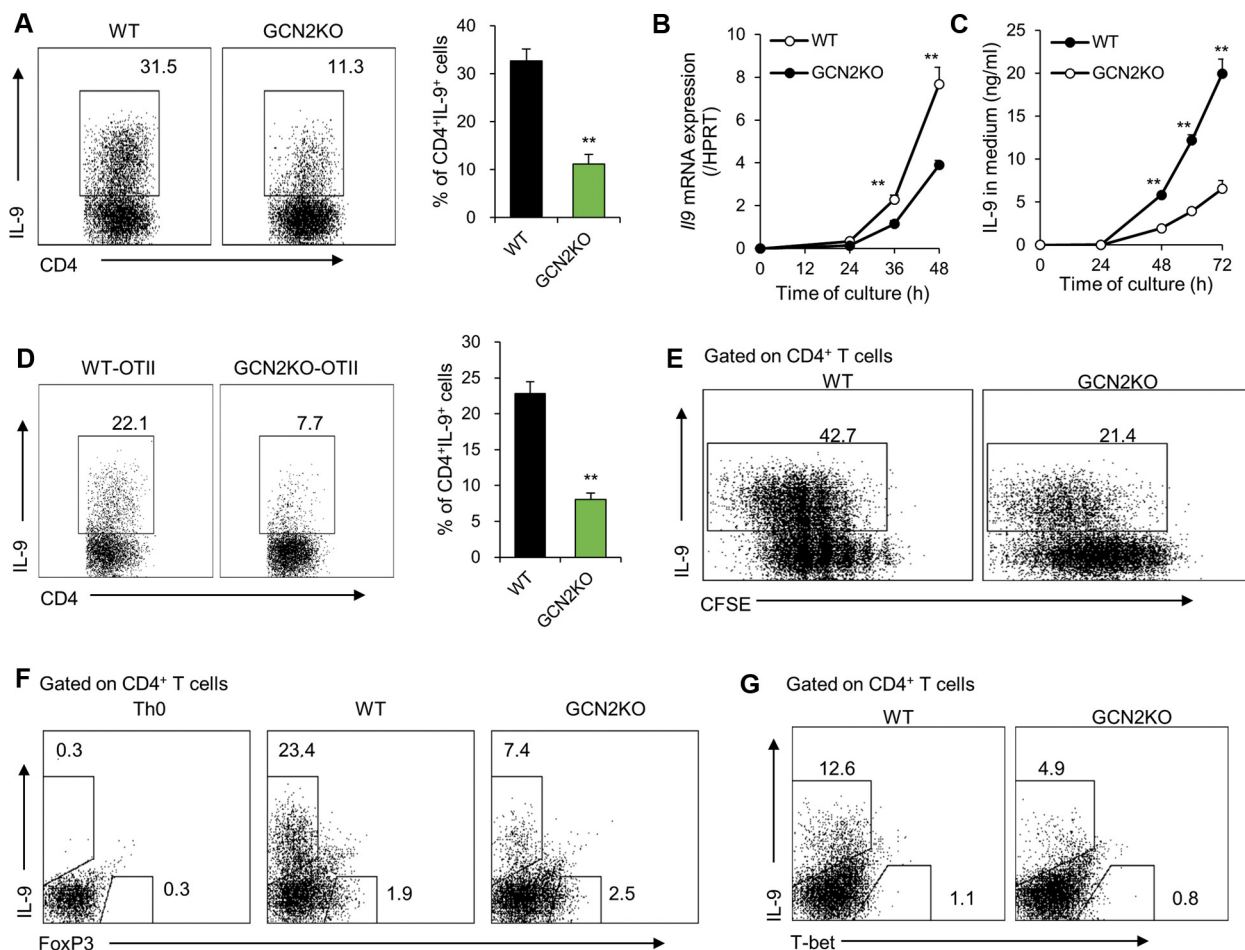


FIG 1. GCN2 is essential for T_H9 cell differentiation *in vitro*. **A**, Percentages of CD4⁺IL-9⁺ T_H9 cells in naive CD4⁺ T cells of WT and GCN2KO mice under T_H9-skewing conditions. **B**, Expression of *Il9* mRNA in naive WT and GCN2KO CD4⁺ T cells during the process of T_H9 cell differentiation. **C**, IL-9 production in naive WT and GCN2KO CD4⁺ T cells during the process of T_H9 cell differentiation. **D**, Percentages of CD4⁺IL-9⁺ T cells in naive CD4⁺ T cells of WT-OT-II and GCN2KO-OT-II mice stimulated with OVA₃₂₃₋₃₃₉ peptide under T_H9 induction conditions. **E**, Percentages of CD4⁺IL-9⁺ carboxyfluorescein succinimidyl ester (CFSE)⁺ T cells in naive CD4⁺ T cells of WT and GCN2KO mice under T_H9-skewing conditions. **F**, Intracellular staining of IL-9 and Foxp3 in CD4⁺ T cells of WT and GCN2KO mice cultured under T_H9-skewing conditions. **G**, Intracellular staining of IL-9 and T-bet in CD4⁺ T cells of WT and GCN2KO mice cultured under T_H9-skewing conditions. Data are shown as means $\pm$ SDs (n = 3), representing one of 3 independent experiments. **P < .01.

GCN2KO mice (see Fig E7, B). Histologic analysis of lung sections using H&E staining revealed that lungs of GCN2KO mice have significantly less severe airway inflammation than WT lungs in response to OVA challenge, as indicated by reduced numbers of infiltrated inflammatory cells and pathologic changes (Fig 2, B, and see Fig E8 in this article's Online Repository at www.jacionline.org). Consistently, total cell numbers of infiltrated inflammatory cells in BALF of GCN2KO mice were also significantly lower than those of WT mice (P < .01; Fig 2, C).

As the major cell type of BALF cells, eosinophils play a critical role in OVA-induced pulmonary inflammation. We found that GCN2 deficiency significantly decreased numbers of eosinophils in BALF from OVA-challenged mice (P < .01; Fig 2, C) but had no effect on neutrophils, macrophages, and lymphocytes. OVA treatment resulted in greater percentages of CD11b⁺Siglec-F⁺

eosinophils in BALF cells from WT mice than those of GCN2KO mice (67.0% $\pm$ 2.6% vs 24.34% $\pm$ 8.0%, P < .01; Fig 2, D).

Mucus hypersecretion in pulmonary bronchus is a feature of allergic inflammation in the lungs.³⁸ As revealed by PAS staining of lung tissues, GCN2KO lungs produced less mucus in bronchus than WT lungs in response to OVA treatment (Fig 2, E, and see Fig E8). OVA challenge also increased MCPT-1 levels in the sera of WT mice than those seen in GCN2 mice (P < .01; Fig 2, F), which is a marker of the infiltration of mast cells in trachea.¹⁴ Furthermore, there were fewer mast cells in lungs of GCN2KO mice than those of WT mice (see Fig E9 in this article's Online Repository at www.jacionline.org).

To investigate the effects of GCN2 on T_H9 cell differentiation and IL-9 expression *in vivo*, we measured T_H9 cell percentages and IL-9 production in lungs from OVA-challenged mice. The

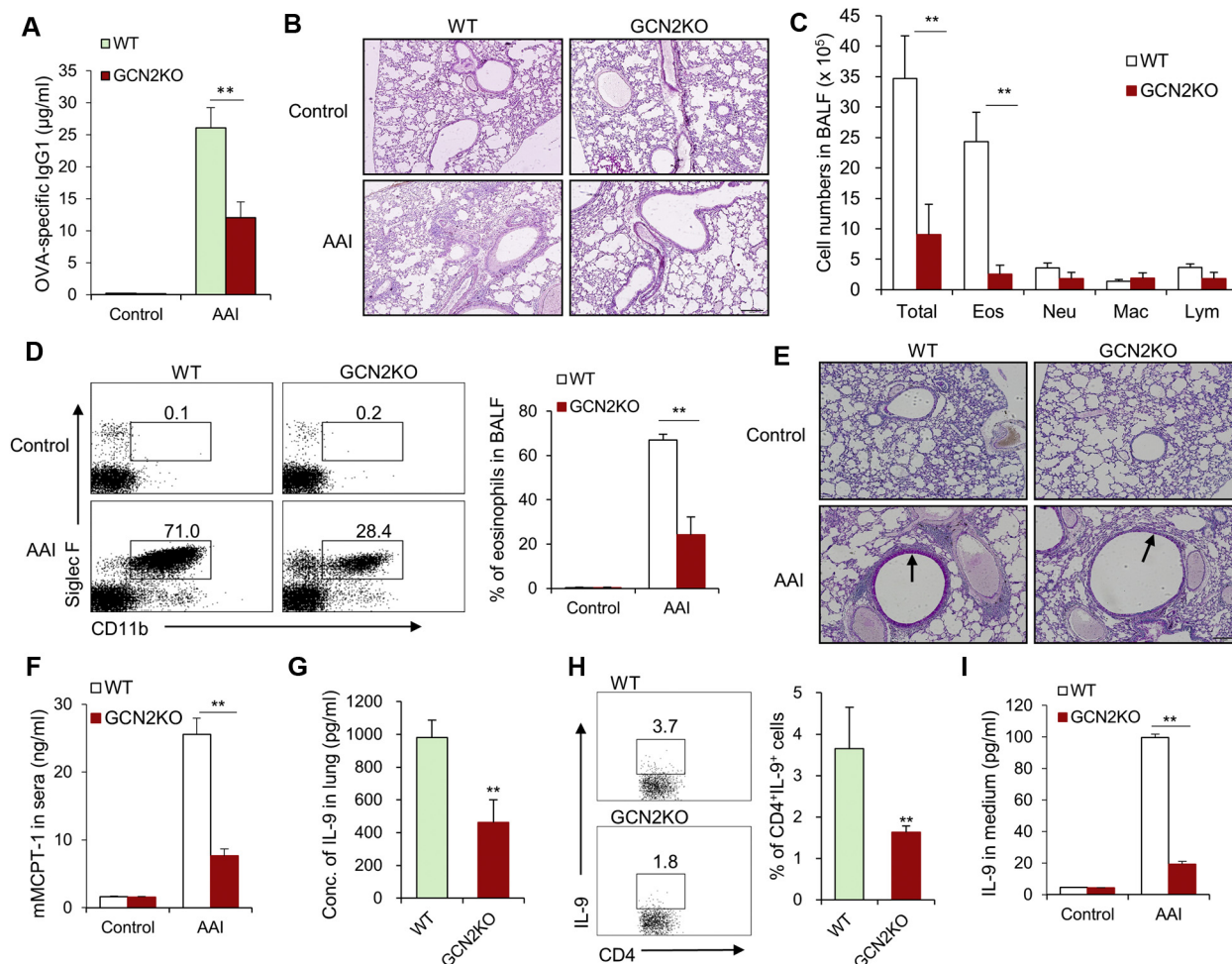


FIG 2. GCN2-deficient mice are resistant to allergic airway inflammation. An OVA-induced allergic airway inflammation model (AAI) was carried out in WT and GCN2KO mice. **A**, Levels of OVA-specific IgG₁ in the sera of WT and GCN2KO mice (n = 4-6 per group). **B**, H&E staining of lung tissues of WT and GCN2KO mice. **C**, Cell numbers of immunocytes in BALF of WT and GCN2KO mice. *Eos*, Eosinophils; *Lym*, lymphocytes; *Mac*, macrophages; *Neu*, neutrophils. **D**, Percentages of eosinophils in BALF of WT and GCN2KO mice. **E**, PAS staining of lung tissues of WT and GCN2KO mice. **F**, Mouse MCPT-1 levels in sera of WT and GCN2KO mice. **G**, Concentrations of IL-9 in homogenates of lung tissues of WT and GCN2KO mice. **H**, Percentages of CD4⁺IL-9⁺ cells in WT and GCN2KO mice. **I**, IL-9 production in supernatants of dLNs of WT and GCN2KO mice (n = 4-6 per group) stimulated with OVA for 3 days. Data represent 3 independent experiments. Data are shown as means ± SDs. **P < .01.

results showed that pulmonary IL-9 production in GCN2KO mice was significantly less than that in WT mice among OVA-challenged mice ($P < .01$; Fig 2, G). Isolated cells from dLNs were stimulated with PMA and ionomycin to detect CD4⁺IL-9⁺ T_H9 cells. Consistent with the decreased pulmonary IL-9 production in GCN2KO mice, percentages of CD4⁺IL-9⁺ T_H9 cells in dLNs of GCN2KO mice were significantly lower than those in WT mice in the OVA-induced allergic airway inflammation model ($P < .01$; Fig 2, H). After stimulation with OVA for 3 days, cells isolated from dLNs of OVA-treated GCN2KO mice produced significantly less IL-9 in supernatant than those in WT mice, as detected by means of ELISA ($P < .01$; Fig 2, I).

Together, our results suggest that GCN2 deficiency attenuated T_H9 cell differentiation and IL-9 production *in vivo* and alleviated allergic inflammation in the lungs in the OVA-induced allergic airway inflammation model.

GCN2 deficiency in CD4⁺ T cells alleviates allergic airway inflammation

It is known that GCN2 has wide effects on immune cells, such as CD11c⁺ and Treg cells.²⁰⁻²³ To determine whether GCN2-deficient CD4⁺ T cells could alleviate allergic inflammation, we adoptively transferred WT and GCN2KO CD4⁺ T cells to syngeneic Rag2KO mice and then subjected the mice to the process of OVA-induced allergic airway inflammation. Both types of Rag2KO mice received sorted WT or GCN2KO CD4⁺ T cells had similar percentages of CD4⁺ T cells in peripheral blood after these cells were transferred for 7 days (Fig 3, A), indicating that the GCN2 deficiency does not affect the reconstitution of transferred CD4⁺ T cells in immune-deficient Rag2KO mice. However, H&E staining of lung sections showed that Rag2KO mice that received the sorted GCN2KO CD4⁺ T cells (GCN2KO-CD4⁺ T → Rag2KO) had alleviated allergic inflammation compared with Rag2KO mice

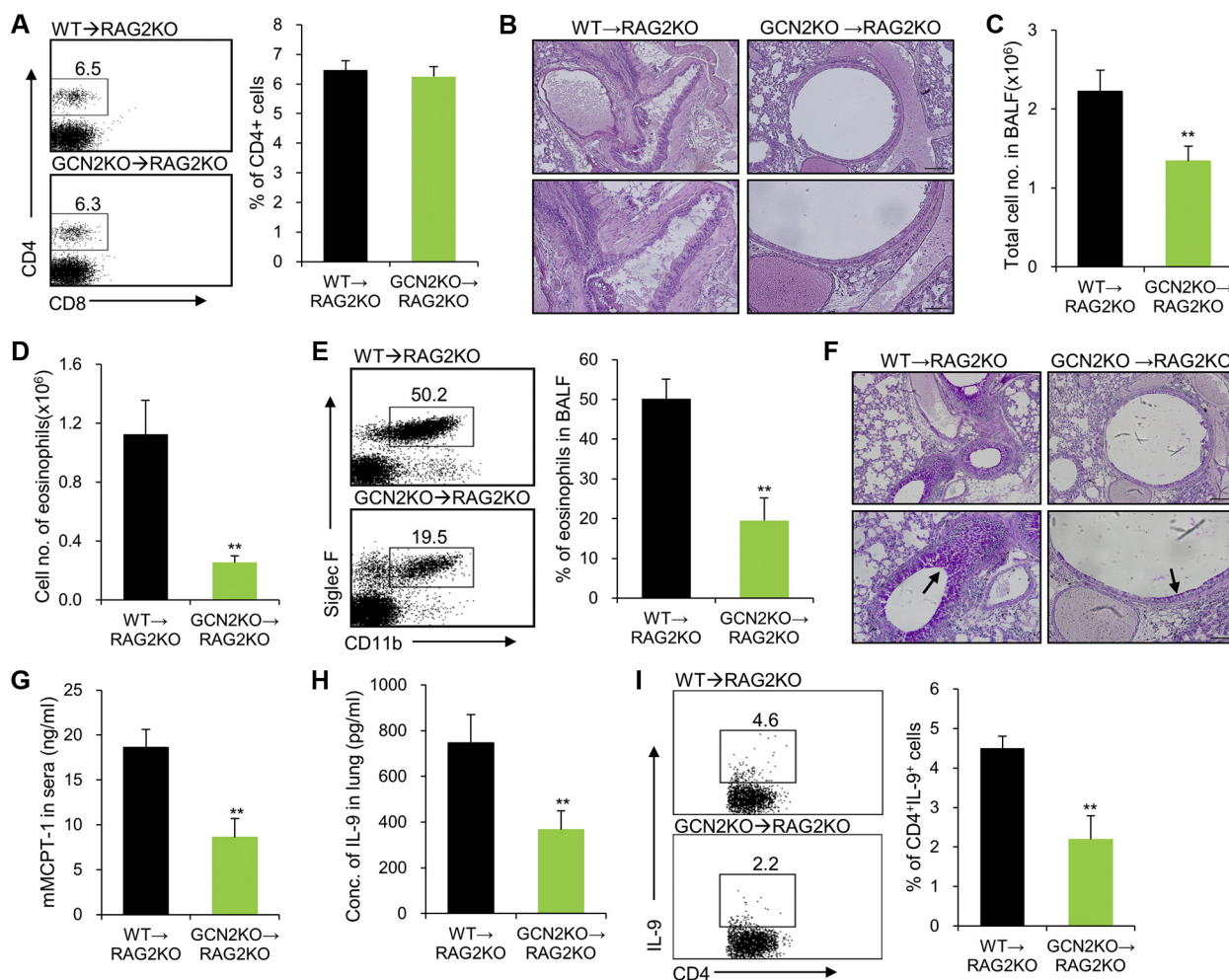


FIG 3. GCN2-deficient CD4⁺ T cells mediate less severe allergic inflammation. An OVA-induced allergic airway inflammation model was carried out on Rag2KO mice that received WT and GCN2KO CD4⁺ T cells. **A**, Percentages of CD4⁺ T cells in Rag2KO mice received sorted CD4⁺ T cells (WT→Rag2KO) and GCN2KO CD4⁺ T cells (GCN2KO→Rag2KO). **B**, Lung tissue H&E staining of allergic inflammatory Rag2KO mice that received WT and GCN2KO CD4⁺ T cells. Original magnification ×50 (top row) or ×100 (bottom row). **C**, Total cell numbers in BALF of Rag2KO mice that received WT and GCN2KO CD4⁺ T cells (n = 5 per group). **D**, Eosinophil numbers in BALF of Rag2KO mice that received WT and GCN2KO CD4⁺ T cells (n = 5 per group). **E**, Percentages of eosinophils in BALF of Rag2KO mice that received WT and GCN2KO CD4⁺ T cells in an OVA-induced allergic airway inflammation model (n = 5 per group). **F**, Lung tissue PAS staining of Rag2KO mice that received WT and GCN2KO CD4⁺ T cells. Original magnification ×50 (top row) or ×100 (bottom row). **G**, Mouse MCPT-1 levels in the sera of Rag2KO mice that received WT and GCN2KO CD4⁺ T cells. **H**, Concentrations of IL-9 in homogenates of lung tissues of Rag2KO mice received WT and GCN2KO CD4⁺ T cells. **I**, Percentages of CD4⁺IL-9⁺ cells of dLNs of Rag2KO mice received WT and GCN2KO CD4⁺ T cells (n = 5 per group). Data (means ± SDs) represent one of 3 independent experiments. ***P* < .01.

that received sorted WT CD4⁺ T cells (WT-CD4⁺ T→Rag2KO; Fig 3, B, and see Fig E10 in this article's Online Repository at www.jacionline.org). Meanwhile, total cell numbers of infiltrated inflammatory cells in BALF of GCN2KO-CD4⁺ T→Rag2KO mice were significantly lower than those of WT-CD4⁺ T→Rag2KO mice (*P* < .01; Fig 3, C). Both numbers and proportions of infiltrated eosinophils were significantly lower in GCN2KO-CD4⁺ T→Rag2KO mice than those in WT-CD4⁺ T→Rag2KO mice (*P* < .01; Fig 3, D and E). Histologic analysis of lung tissues showed that there was significantly less mucus production in bronchi of GCN2KO-CD4⁺ T→Rag2KO mice than that in bronchi of WT-CD4⁺ T→Rag2KO mice (Fig 3, F, and see Fig E10). Consistently, levels of MCPT-1 in peripheral

blood of GCN2KO-CD4⁺ T→Rag2KO mice were also significantly lower than those of WT-CD4⁺ T→Rag2KO mice (*P* < .01; Fig 3, G). Meanwhile, there were fewer mast cells in the lungs of GCN2KO-CD4⁺ T→Rag2KO mice than those of WT-CD4⁺ T→Rag2KO mice (see Fig E11 in this article's Online Repository at www.jacionline.org). Furthermore, significantly less IL-9 production in the lungs of GCN2KO-CD4⁺ T→Rag2KO mice than in the lungs of WT-CD4⁺ T→Rag2KO mice was observed after OVA challenge (*P* < .01; Fig 3, H). Similarly, percentages of CD4⁺IL-9⁺ T_H9 cells were significantly lower in dLNs of GCN2KO-CD4⁺ T→Rag2KO mice than those of WT-CD4⁺ T→Rag2KO mice (*P* < .01; Fig 3, I). Therefore GCN2 deficiency in CD4⁺ T cells decreased

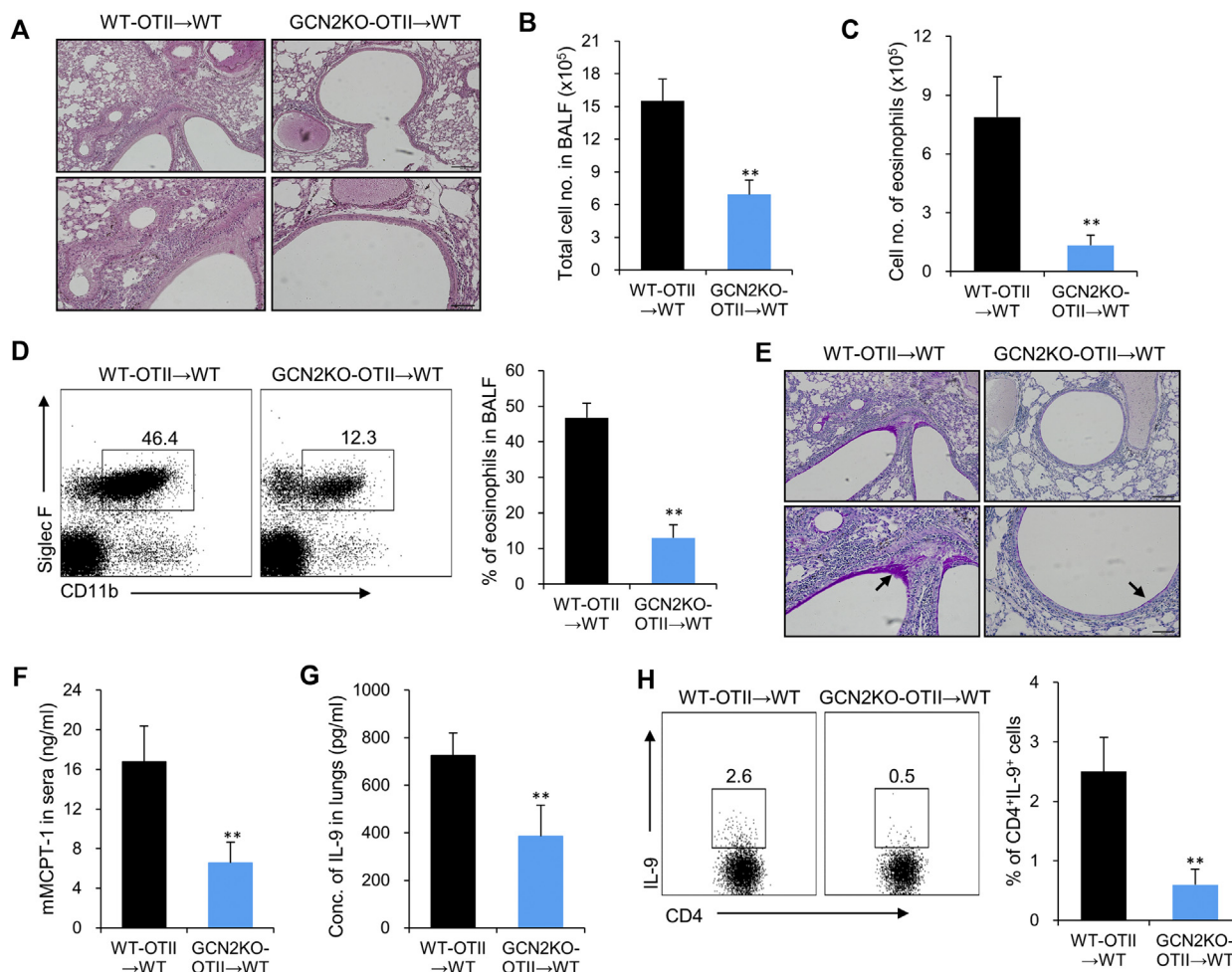


FIG 4. GCN2-deficient T_H9 cells mediate less severe allergic inflammation on adoptive transfer. Naive WT-OT-II and GCN2KO-OT-II $CD4^+$ T cells were polarized *in vitro* under T_H9 conditions and transferred into syngeneic WT recipients ($n = 4-6$ per group) before daily exposure to OVA aerosol for 5 days and subsequent analysis of pulmonary inflammation. **A**, H&E staining of lung tissues of recipients. **B**, Total cell numbers in BALF of recipients. **C**, Total eosinophil cell numbers in BALF of recipients. **D**, Percentages of eosinophil in BALF of recipients. Original magnification $\times 50$ (top row) or $\times 100$ (bottom row). **E**, PAS staining of lung tissues of recipient mice. Original magnification $\times 50$ (top row) or $\times 100$ (bottom row). **F**, Mouse MCPT-1 levels in sera of recipients. **G**, Concentrations of IL-9 in homogenates of lung tissues of recipients. **H**, Percentages of $CD4^+IL-9^+$ T cells of BALF of recipients. Data (means $\pm$ SDs) represent one of 3 independent experiments. $**P < .01$.

T_H9 cell differentiation and IL-9 production *in vivo* and was sufficient to alleviate the allergic airway inflammation.

GCN2-deficient T_H9 cells mediate less severe allergic inflammation

To further determine whether attenuated T_H9 cell differentiation contributes to ameliorated allergic airway inflammation in GCN2KO mice, we used an allergic airway inflammation mouse model through adoptive transfer of *in vitro*-induced OVA-specific OT-II T_H9 cells. WT B6 mice were adoptively transferred with *in vitro*-induced WT-OT-II or GCN2KO-OT-II T_H9 cells and then challenged with aerosolized OVA without preimmunization. Mice receiving GCN2KO-OT-II T_H9 cells produced significantly lower levels of OVA-specific IgE in sera than mice receiving WT-OT-II T_H9 cells ($P < .01$, see Fig E12 in this article's Online Repository at www.jacionline.org). As demonstrated by using

H&E staining, lung sections from mice that received GCN2KO-OT-II T_H9 cells exhibited obviously alleviated allergic airway inflammation compared with those of mice that received WT-OT-II T_H9 cells (Fig 4, A, and see Fig E13 in this article's Online Repository at www.jacionline.org). Consistently, infiltrated total numbers of immunocytes and eosinophils in BALF of mice that received GCN2KO-OT-II T_H9 cells were significantly lower than those of mice that received WT-OT-II T_H9 cells ($P < .01$; Fig 4, B and C), which also showed alleviated allergic airway inflammation in mice that received GCN2KO-OT-II T_H9 cells. Moreover, percentages of infiltrated eosinophils in BALF of mice that received GCN2KO-OT-II T_H9 cells were also significantly lower than those of mice that received WT-OT-II T_H9 cells ($P < .01$; Fig 4, D). Furthermore, PAS staining of lung tissues showed that mucus production in pulmonary bronchus of mice that received GCN2KO-OT-II T_H9 cells was distinctly less than those of mice that received

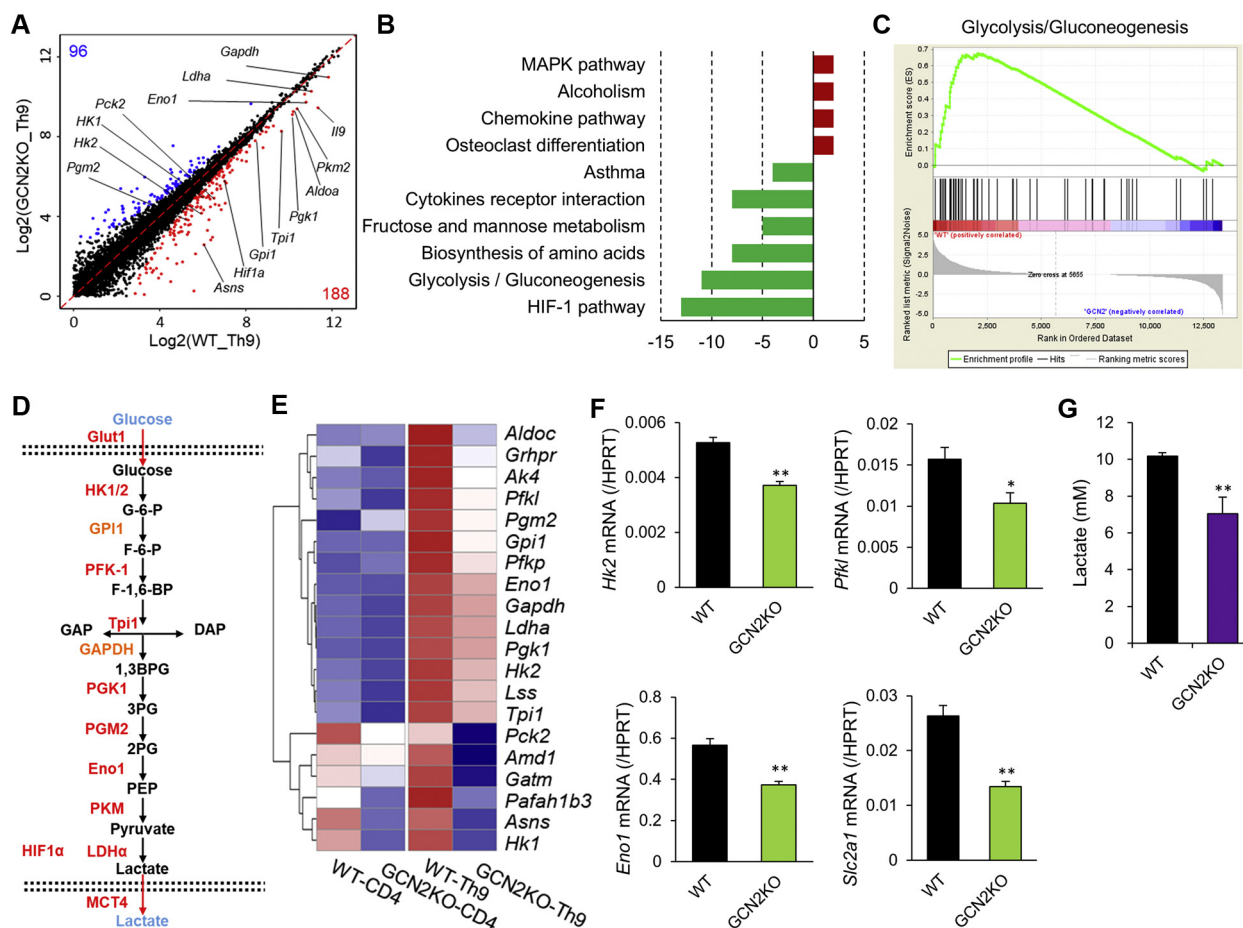


FIG 5. GCN2 regulates TH9 cell differentiation through the glycolytic pathway. **A**, Scatter plots of differential genes between WT and GCN2KO TH9 cells. Colors are restricted by the principle coming from a *P* value of less than .05 and a fold change of greater than |1.5|. **B**, KEGG signal pathway enrichment of differential genes between WT and GCN2KO TH9 cells. The top 10 terms were shown. **C**, Gene set enrichment analysis plot presents downregulated DEGs in GCN2KO TH9 cells enriched in glycolytic pathway. **D**, Diagram of all downregulated molecules in the glycolytic pathway. Red represents decreasing fold change of greater than 2, and orange represents decreasing fold change of greater than 1.5 in GCN2KO mice. **E**, Heat map represents expression of the corresponding genes in the glycolysis process. **F**, Expression of *Hk2*, *Pfkf*, *Eno1*, and *Slc2a1* mRNA in WT and GCN2KO TH9 cells. **G**, Production of lactate in naive WT and GCN2KO CD4⁺ T cells under TH9 induction conditions. Data are shown as means $\pm$ SDs (*n* = 3), representing one of at least 2 independent experiments. **P* < .05 and ***P* < .01.

WT-OT-II TH9 cells (Fig 4, E, see Fig E13). Meanwhile, mice that received GCN2KO-OT-II TH9 cells also exhibited significantly lower serum MCPT-1 levels and fewer mast cells in lungs than mice that received WT-OT-II TH9 cells (*P* < .01; Fig 4, F; see Fig E14 in this article's Online Repository at www.jacionline.org).

Next, we measured IL-9 content in lung tissues and found that the pulmonary IL-9 levels in mice that received GCN2KO-OT-II TH9 cells was significantly lower than that in mice that received WT-OT-II TH9 cells (Fig 4, G). Similarly, there were significantly lower percentages of CD4⁺IL-9⁺ TH9 cells in BALF of mice that received GCN2KO-OT-II TH9 cells than those of mice that received WT-OT-II TH9 cells (*P* < .01; Fig 4, H). However, there was no difference in percentages of CD4⁺Foxp3⁺ T cells in BALF of mice that received GCN2KO-OT-II TH9 cells or WT-OT-II TH9 cells (see Fig E15 in this article's Online Repository at www.jacionline.org). Together, these results suggest that the defect of TH9 cell differentiation and function

caused by GCN2 deficiency remarkably attenuated allergic airway inflammation in mice.

GCN2 regulates TH9 cell differentiation through the glycolytic pathway

After the critical effect of GCN2 on TH9 cell differentiation was verified both *in vitro* and *in vivo*, we tried to investigate the molecular mechanisms by which GCN2 positively regulates TH9 cell differentiation. To screen the changed genes and pathways associated with TH9 cell differentiation caused by GCN2 deficiency, we conducted RNA sequencing (RNA-Seq) analysis of induced TH9 cells from WT and GCN2-deficient CD4⁺ T cells. In the 284 significantly changed genes, there were 96 genes upregulated and 188 genes downregulated in induced TH9 cells from GCN2-deficient naive CD4⁺ T cells when compared with induced TH9 cells from WT naive CD4⁺ T cells (Fig 5, A).

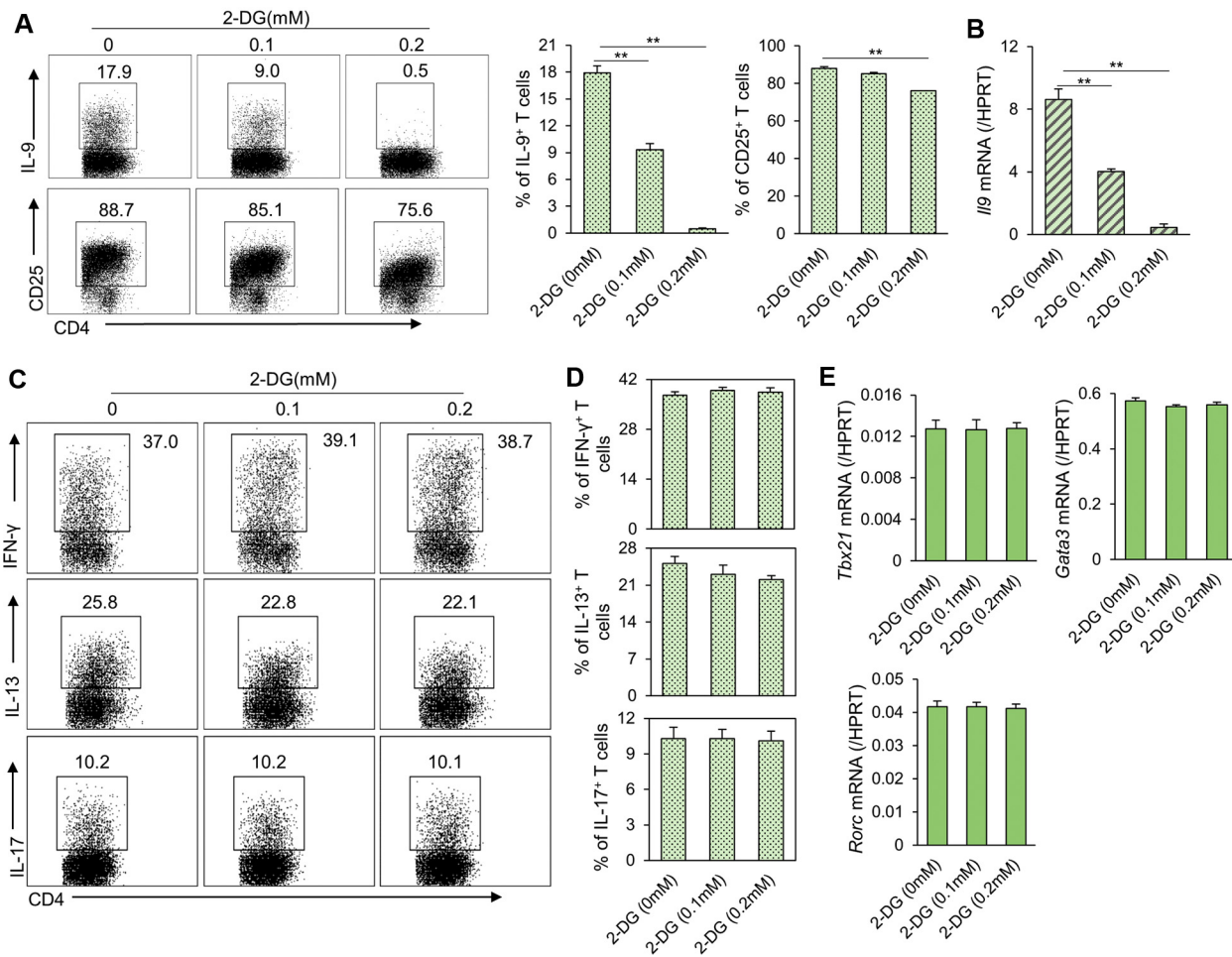


FIG 6. Low doses of 2-DG decreased T_H9 cell differentiation. **A**, Percentages of CD4⁺IL-9⁺ T_H9 cells under T_H9 induction conditions simultaneously treated with 2-DG. **B**, Expression of *Il9* mRNA in naive CD4⁺ T cells treated with 2-DG under T_H9 induction conditions. **C** and **D**, Flow cytometry (Fig 5, C) and percentages (Fig 5, D) of CD4⁺IFN-γ⁺, CD4⁺IL-13⁺, and CD4⁺IL-17⁺ T cells in naive CD4⁺ T cells of WT and GCN2KO mice under T_H1, T_H2, and T_H17 induction conditions, respectively, simultaneously treated with 2-DG. **E**, Expression of *Tbx21*, *Gata3*, and *Rorc* in naive CD4⁺ T cells of WT and GCN2KO mice under T_H1, T_H2, and T_H17 induction conditions, respectively, simultaneously treated with 2-DG. mRNA expression was detected by using real-time PCR. Data are shown as means ± SDs (n = 3), representing one of at least 2 independent experiments. **P < .01.

To further determine which signaling pathways were regulated by GCN2 deficiency in the process of T_H9 cell differentiation, these significantly changed genes were further investigated by using KEGG analysis. The results showed that downregulated genes were mainly enriched in the HIF-1 pathway and the glycolysis/gluconeogenesis and biosynthesis of amino acids pathways, whereas upregulated genes were not primarily concentrated in one pathway (Fig 5, B). Consistently, gene set enrichment analysis also showed that downregulated genes were significantly enriched in the glycolysis/gluconeogenesis pathway (Fig 5, C). Impressively, expression of all genes encoding rate-limiting enzymes in the glycolytic pathway was significantly decreased in induced GCN2KO T_H9 cells, such as *HK1*, *HK2*, *PFK-1*, and *PKM* (Fig 5, D and E). Meanwhile, expression of *Slc2a1*, the gene encoding glucose transporter 1, and *Slc16a3*, the gene encoding monocarboxylate transporter 4, was also decreased in induced GCN2KO T_H9 cells (Fig 5, D and E). Real-time PCR results confirmed the significantly

decreased expression of these key genes, such as *Hk2*, *Pfkfb1*, *Eno1*, and *Slc2a1*, as shown by using RNA-Seq analysis (Fig 5, F). In parallel, lactate production in supernatants of GCN2-deficient naive CD4⁺ T cells under T_H9-skewing conditions was also significantly lower than that of WT CD4⁺ naive T cells (P < .01; Fig 5, G), which also suggested that GCN2 deficiency reduced glycolytic metabolism during T_H9 cell differentiation.

It has been demonstrated that activated T cells primarily rely on aerobic glycolysis to maintain the energy requirement for proliferation and differentiation.³⁹ Therefore these results suggest that the glycolytic metabolism pathway altered by GCN2 deficiency might be involved in regulation of GCN2 on T_H9 cell differentiation. Thus we determined the effects of 2-deoxy-d-glucose (2-DG), a glycolysis inhibitor, on differentiation of different subtypes of T_H cells. We found that 0.1 mmol/L 2-DG significantly reduced generation of CD4⁺IL-9⁺ T_H9 cells, whereas it did not affect expression of

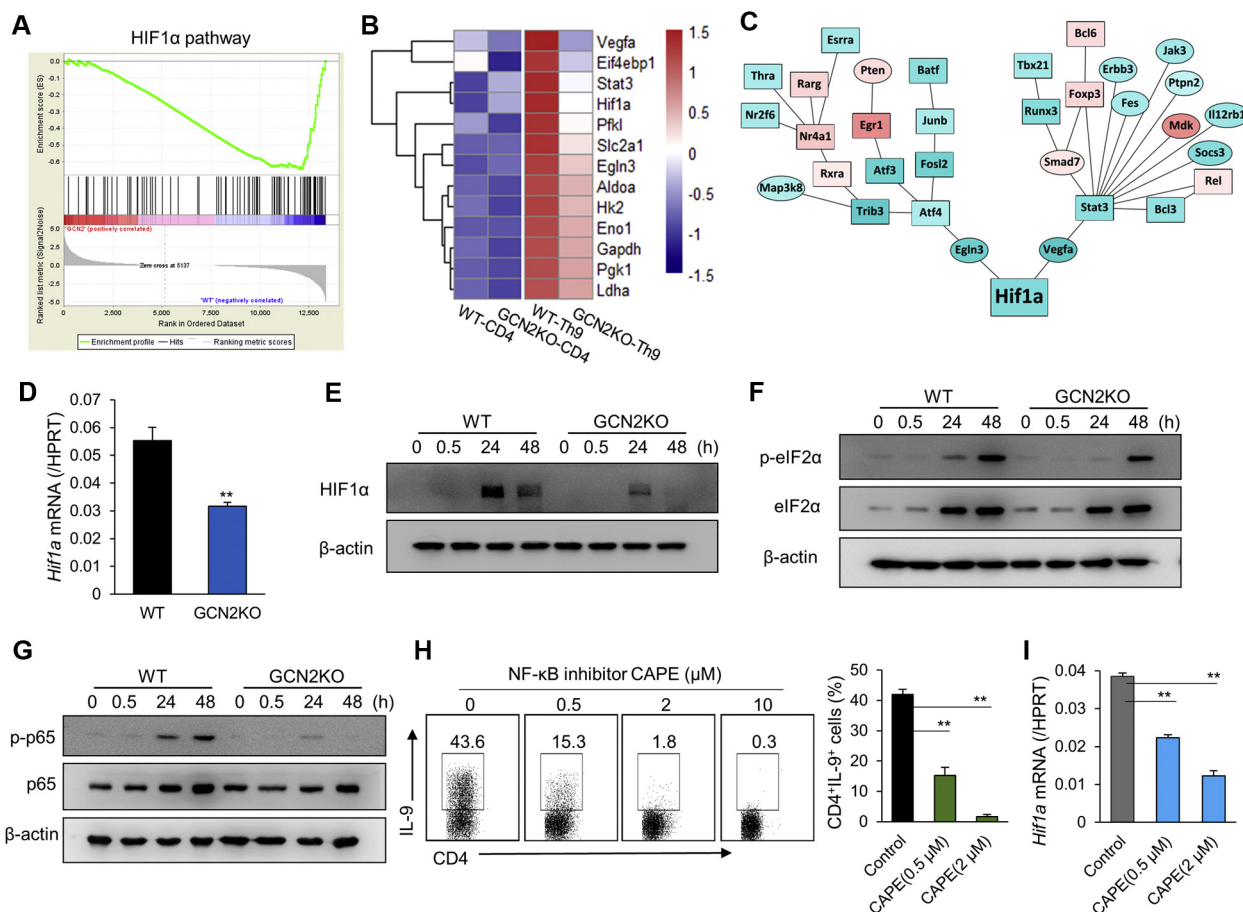


FIG 7. GCN2 regulates TH9 cell differentiation through the NF-κB-HIF1α signaling pathway. **A**, Gene set enrichment analysis plot showing that downregulated DEGs in GCN2KO TH9 cells were enriched in the HIF1α signaling pathway. **B**, Heat map of altered genes involved in the HIF1α signaling pathway in WT and GCN2KO TH9 cells. **C**, Interaction network of transcription factors in DEGs (fold change > 1.5), as visualized by using Cytoscape. **D**, Expression of *Hif1a* mRNA in WT and GCN2KO TH9 cells. **E**, Immunoblot analysis of HIF1α and β-actin in CD4⁺ T cells of WT and GCN2KO mice under TH9 induction conditions for the indicated time points. **F**, Immunoblot analysis of phosphorylated eIF2α, eIF2α, and β-actin in WT and GCN2KO CD4⁺ T cells under TH9 induction conditions. **G**, Immunoblot analysis of phosphorylated p65, p65, and β-actin in CD4⁺ T cells of WT and GCN2KO mice under TH9 induction conditions for the indicated time points. **H**, Percentages of CD4⁺IL-9⁺ TH9 cells treated with the NF-κB inhibitor caffeic acid phenethyl ester (CAPE) under TH9 induction conditions. Data represent one of at least 2 independent experiments. **I**, Expression of *Hif1a* mRNA treated with the NF-κB inhibitor CAPE under TH9 induction conditions. Data are shown as means ± SDs (n = 3), representing one of at least 2 independent experiments. **P < .01.

the T-cell activation marker CD25 under TH9 induction conditions (Fig 6, A). 2-DG (0.2 mmol/L) further reduced generation of CD4⁺IL-9⁺ TH9 cells and slightly decreased CD25 expression ($P < .01$; Fig 6, A). Consistently, a low dose of 2-DG (0.1-0.2 mmol/L) also significantly decreased *Ii9* mRNA expression ($P < .01$; Fig 6, B). However, 0.1 to 0.2 mmol/L 2-DG did not affect the generation of CD4⁺IFN-γ⁺ TH1 cells, CD4⁺IL-4⁺ TH2 cells, and CD4⁺IL-17⁺ TH17 cells under TH1, TH2, and TH17 induction conditions, respectively (Fig 6, C). Meanwhile, low doses of 2-DG did not affect expression of the master transcription factors of TH1, TH2, and TH17 cells, including *Tbx21*, *Gata3*, and *Rorc* (Fig 6, D). Thus TH9 cell differentiation seems more sensitive to changes in glycolytic metabolism than other subtypes of TH cells, and GCN2 masters TH9 cell differentiation through regulation of glycolytic metabolism.

GCN2 regulates TH9 cell differentiation through the NF-κB-HIF1α signaling pathway

Both KEGG and gene set enrichment analysis showed that the HIF1α pathway was significantly decreased in GCN2-deficient TH9 cells compared with WT TH9 cells (Figs 5, B, and 7, A) and that altered genes involved in the HIF1α signaling pathway were summarized in Fig 7, B. We analyzed the network of differentially associated transcription factors of TH9 cells in WT and GCN2KO TH9 cells and found that the transcription factor HIF1α was in the core of the signal networks (Fig 7, C).

Subsequently, we measured HIF1α expression both at the mRNA and protein levels and found that expression of HIF1α in GCN2-deficient CD4⁺ T cells was significantly lower than that in WT CD4⁺ T cells during the process of TH9 cell differentiation (Fig 7, D and E). HIF1α has been widely recognized as a transcription factor orchestrating expression of glycolytic

enzymes,^{40,41} and the HIF1 α -dependent glycolytic signaling pathway could promote T_H9 cell differentiation through directly binding to the *Ii9* promoter to promote IL-9 transcription.⁴² Thus GCN2 regulates T_H9 cell differentiation likely through an HIF1 α -dependent glycolytic pathway and the direct regulatory effect of HIF1 α on *Ii9* mRNA expression.

The next question is how GCN2 regulates HIF1 α during T_H9 cell differentiation. It has been reported that there is an NF- κ B binding site in the promoter of HIF1 α , and NF- κ B activation directly promotes HIF1 α transcription.^{43–47} It has also been reported that GCN2-mediated phosphorylation of eIF2 α could activate NF- κ B.^{48–50} Hence we speculated that GCN2 regulates HIF1 α expression through phosphorylation of eIF2 α and activation of the NF- κ B signaling pathway. As expected, the level of phosphorylation of eIF2 α in GCN2-deficient CD4⁺ T cells was significantly lower than that in WT CD4⁺ T cells under T_H9 induction conditions (Fig 7, F). Meanwhile, levels of p65 phosphorylation, which represents NF- κ B activation, in GCN2-deficient CD4⁺ T cells were also markedly lower than that in WT CD4⁺ T cells under T_H9 conditions (Fig 7, G).

Moreover, to confirm the effect of NF- κ B on T_H9 cell differentiation, we treated the sorted WT CD4⁺ T cells with an NF- κ B inhibitor (caffeic acid phenethyl ester) during the process of T_H9 cell differentiation. The NF- κ B inhibitor significantly reduced production of CD4⁺IL-9⁺ T_H9 cells and expression of *Ii9* mRNA in a dose-dependent manner ($P < .01$; Fig 7, H, and see Fig E16 in this article's Online Repository at www.jacionline.org). Meanwhile, NF- κ B inhibitor treatment significantly decreased mRNA expression of *Hif1 α* ($P < .01$; Fig 7, I), which confirmed that HIF1 α could be regulated by NF- κ B during T_H9 differentiation.

In addition, we also analyzed the expression of other T_H9 differentiation-associated molecules and transcription factors, including STAT6, PU.1, and IRF4, using Western blotting. The results showed there were no obvious differences in the activation of IL-4 downstream STAT6 and TGF- β downstream Smad2 between WT and GCN2KO T_H9 cells (see Fig E17 in this article's Online Repository at www.jacionline.org). Meanwhile, there was no detectable change in BATF expression in WT and GCN2KO T_H9 cells (see Fig E17, B). However, both PU.1 and IRF4 were slightly decreased in GCN2KO T_H9 cells compared with WT T_H9 cells (see Fig E17, B). We speculated that the slight downregulation of IRF4 and PU.1 in GCN2KO T_H9 cells can be caused by the decreased HIF1 α -dependent glycolytic metabolism because 2-DG treatment decreased expression of PU.1 and IRF4 in T_H9 cells (see Fig E18 in this article's Online Repository at www.jacionline.org). Taken together, these results indicate that GCN2 promotes T_H9 cell differentiation mainly through the eIF2 α -NF- κ B-HIF1 α pathway and its relevant glycolytic metabolism.

DISCUSSION

The present study revealed 2 new major findings. First, we demonstrated that deficiency of the amino acid sensor GCN2 selectively inhibits T_H9 differentiation and IL-9 production, which leads to less severe allergic inflammation in the allergic airway inflammation mouse model. Second, the attenuated T_H9 differentiation in naive GCN2KO CD4⁺ T cells was predominantly caused by downregulation of the eIF2 α -NF- κ B-HIF1 α pathway and its mediated glycolytic pathway.

CD4⁺ T cells are an important component of adaptive immunity and can differentiate into various T_H cell subsets under different cytokine microenvironments. As an amino acid sensor, GCN2 has been shown to affect T_H cell differentiation. For instance, tryptophanol, a GCN2 activator, decreased expression of GATA-3, a marker for T_H2 cell differentiation, in CD4⁺ T cells isolated from mixed lymphocyte reactions.⁵¹ Indoleamine 2,3-dioxygenase, which activates GCN2 by degrading the essential amino acid tryptophan, was found to promote Treg cell differentiation.⁵² In the present study we found that GCN2 deficiency did not affect naive CD4⁺ T cells to differentiate into CD4⁺IFN- γ ⁺ T_H1 cells, CD4⁺IL-4⁺ T_H2 cells, and CD4⁺Foxp3⁺ iTreg cells under respective induction conditions but selectively attenuated CD4⁺IL-9⁺ T_H9 cell differentiation under T_H9-skewing conditions. Although a previous study suggested that GCN2 in T cells mediates proliferative arrest in response to indoleamine 2,3-dioxygenase,⁵³ we found that GCN2 deficiency did not affect the proliferation ability and activation of CD4⁺ T cells using TCR ligation, as indicated by cell division and expression of activation makers, such as CD69 and CD25,⁵⁴ under T_H0 or T_H cell induction conditions. The finding that OVA-challenged GCN2KO mice had lower pulmonary IL-9 levels and percentages of CD4⁺IL-9⁺ T_H9 cells in dLNs suggests that GCN2 deficiency could impair T_H9 cell differentiation *in vivo*. This was further confirmed in the CD4⁺ T cell-transferred allergic airway inflammation model, in which we demonstrated that adoptively transferred GCN2KO CD4⁺ T cells into Rag2KO mice resulted in lower percentages of CD4⁺IL-9⁺ T_H9 cells in dLNs of GCN2KO-CD4⁺T $\rightarrow$ Rag2KO mice after OVA challenge. Taken together, our data identified a novel intrinsic role for GCN2 in regulating T_H9 cell differentiation.

Recently, dysregulation of T_H9 cells has been observed in patients with various diseases, especially allergic asthma.⁶ T_H9 cell numbers and IL-9 production in children with atopy were significantly greater than those in healthy subjects.⁸ Adoptive transfer of T_H9 cells promotes pulmonary inflammation by accumulating eosinophils and mast cells and increasing mucus, antigen-specific IgE, and T_H2-associated cytokine production.^{7,14,15,29} By contrast, IL-9 neutralization or IL-9-deficient T_H9 cells transfer effectively alleviated bronchial hyperresponsiveness and pulmonary inflammation.^{7,14,15,29} Therefore it is likely that the impaired T_H9 cell differentiation and reduced pulmonary IL-9 production caused by GCN2 deficiency might contribute to the attenuated allergic airway inflammatory response in OVA-challenged GCN2KO mice. This hypothesis was supported by the finding that adoptive transfer of GCN2-deficient CD4⁺ T cells to Rag2KO mice resulted in less severe allergic airway inflammation after OVA challenge. Furthermore, mice that received GCN2KO-OT-II T_H9 cells also exhibited lower pulmonary IL-9 levels and less severe allergic airway inflammation than mice that received WT-OT-II T_H9 cells. Thus GCN2 exerts a detrimental role in allergic airway inflammation through promoting CD4⁺ T cells to differentiate into T_H9 cells.

After identifying a novel role for GCN2 in T_H9 cell differentiation and allergic airway inflammation, we studied the underlying molecular mechanisms. It has been demonstrated that the process of CD4⁺ T-cell proliferation and differentiation accompanied metabolic reprogramming.^{55,56} Resting CD4⁺ T cells rely on the tricarboxylic acid cycle (TCA cycle) and

oxidative phosphorylation to provide energy for cell survival. Once activated, these CD4⁺ T cells mainly use glycolysis to support the energy needs of proliferation and differentiation.^{39,57} Meanwhile, effector T cells, including T_H1, T_H2, T_H17, and T_H9 cells, are primarily dependent on glucose uptake and glycolysis to support their proliferation and differentiation, whereas Treg cells mainly rely on fatty acids metabolism.⁵⁷ In these subtypes of T_H cells, T_H9 cells exhibit the greatest glycolytic activity and T_H17 cells take second place, whereas Treg cells exhibited the lowest glycolytic activity.⁴² Our RNA-Seq results showed that GCN2-deficient CD4⁺ T cells under T_H9-skewing conditions significantly decreased expression of HIF1 α and the HIF1 α -dependent glycolytic metabolism pathway compared with that seen in WT CD4⁺ T cells. This observation is consistent with the recent study that demonstrated that the HIF1 α -dependent glycolytic pathway regulates T_H9 cell differentiation and that the transcription factor HIF1 α could directly bind to *Ii9* promoter and promote *Ii9* transcription.⁴² We also found that 0.1 mmol/L of the glycolysis inhibitor 2-DG significantly abolished T_H9 cell differentiation but did not affect other T_H cell subset differentiation. These results further support that T_H9 cells were more sensitive to glycolytic metabolism than other subsets of T_H cells. This might partially explain why decreased GCN2 deficiency-mediated glycolytic metabolism selectively regulates T_H9 cell differentiation but no other T_H subsets.

It has been reported that GCN2 downstream eIF2 α could positively regulate NF- κ B activation and that NF- κ B could directly bind to the *Hif1 α* promoter to promote its transcription.⁴³⁻⁴⁷ Our results show that GCN2 deficiency in CD4⁺ T cells significantly reduced phosphorylation of eIF2 α and p65 under T_H9 induction conditions. Meanwhile, the NF- κ B inhibitor significantly decreased T_H9 cell differentiation *in vitro*. These results collectively indicate that the eIF2 α -NF- κ B-HIF1 α pathway might be involved in regulation of GCN2 on T_H9 cell differentiation.

In addition to HIF1 α , many other transcription factors have also been involved in T_H9 cell differentiation, including PU.1, IRF4, STAT6, Smad2/3, STAT5, and BATF.^{58,59} During the process of T_H9 cell differentiation, we also observed slightly decreased expression of PU.1 and IRF4, 2 master transcription factors of T_H9 cells,^{7,15} in GCN2-deficient CD4⁺ T cells under T_H9-skewing conditions. However, because 2-DG treatment also significantly decreased PU.1 and IRF4 expression under T_H9 induction conditions, it is likely that decreased expression of PU.1 and IRF4 in GCN2-deficient T_H9 cells might not be the direct target of GCN2 during T_H9 cell differentiation but likely a result of decreased HIF1 α -dependent glycolytic metabolism. Therefore GCN2 regulates HIF1 α expression during T_H9 cell differentiation, mainly through phosphorylation of eIF2 α and NF- κ B activation.

In summary, our data provide the first direct evidence that the nutrient sensor GCN2 is a novel regulator of T_H9 cell differentiation and that GCN2-mediated T_H9 cell differentiation contributes greatly to the allergic airway inflammation seen in OVA-challenged mice. GCN2 promotes T_H9 cell differentiation mainly through the eIF2 α -NF- κ B-HIF1 α pathway and its relevant glycolytic pathway. Our results suggest that inhibiting GCN2 activity in CD4⁺ T cells might be a new strategy to treat T_H9-related disorders.

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Key messages

- The amino acid sensor GCN2 specifically and intrinsically regulates T_H9 cell differentiation.
- GCN2 controls T_H9 cell differentiation through the eIF2 α -NF- κ B-HIF1 α pathway and relevant glycolytic metabolism.
- GCN2-deficient mice and mice adoptively transferred GCN2-deficient CD4⁺ T cells showed alleviated allergic airway inflammation with less T_H9 cells *in vivo*.

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Supplementary figure legends

Supplementary Fig. 1. Genotyping of GCN2KO mice and the expression of GCN2.

A, Genotyping of GCN2KO mice. **B**, The expression of *Eif2ak4* mRNA in the sorted CD4⁺ T cells of WT and GCN2KO mice. **C**, Immunoblot analysis of GCN2 and β -actin in the sorted CD4⁺ T cells of WT and GCN2KO mice.

Supplementary Fig. 2. The percentages of peripheral T cells.

A, The percentages of CD4⁺ and CD8⁺ T cells in spleens of WT and GCN2KO mice. **B**, The percentages of CD4⁺ and CD8⁺ T cells in peripheral lymph nodes of WT and GCN2KO mice. **C**, The percentages of naïve CD4⁺CD62L⁺CD44⁻ T cells in spleens of WT and GCN2KO mice.

Supplementary Fig. 3 The proliferation and activation of CD4⁺ T cells.

A, CFSE dilution in WT and GCN2KO CD4⁺ T cells stimulated for 72h with plate-bounded anti-CD3 and soluble anti-CD28 mAbs. The surface expression of activation-associated markers CD25 (**B**) and CD69 (**C**) of WT and GCN2KO naïve CD4⁺ T cells stimulated for 72h with plate-bounded anti-CD3 and soluble anti-CD28 mAbs.

Supplementary Fig. 4. The differentiation of Th cell subsets of WT and GCN2KO naïve CD4⁺ T cells.

A, The expression of *Ifng* mRNA and the percentage of CD4⁺IFN- γ ⁺ T cells in naïve CD4⁺ T cells of WT and GCN2KO mice under Th1-skewing condition. **B**, The expression of *Il-4* mRNA and the percentage of CD4⁺IL-4⁺ T cells in naïve CD4⁺ T cells of WT and GCN2KO mice under Th2-skewing condition. **C**, The expression of *Il-17* mRNA and the percentage of CD4⁺IL-17⁺ T cells in naïve CD4⁺ T cells of WT and GCN2KO mice under Th17-skewing condition. **D**, The expression of *Foxp3* mRNA and the percentage of CD4⁺Foxp3⁺ T cells in naïve CD4⁺ T cells of WT and GCN2KO mice under iTreg-skewing condition. Data are shown as mean $\pm$ SD (n=3), representing one of three independent experiments. **P* < 0.05.

Supplementary Fig. 5. The expressions of master transcription factors in subsets of Th cells.

A, The expression of *Tbx21* in CD4⁺ T cells of WT and GCN2KO mice under Th1 induction condition. **B**, The expression of *Gata3* in CD4⁺ T cells of WT and GCN2KO mice under Th2 induction condition. **C**, The expression of *Rorc* in CD4⁺ T cells of WT and GCN2KO mice under Th17 induction condition.

Supplementary Fig. 6. Double intracellular staining of cytokines in CD4⁺ T cells of WT and GCN2KO mice under Th9 induction condition.

Intracellular staining of IL-9 and IFN- γ (**A**), IL-9 and IL-4 (**B**), IL-9 and IL-17 (**C**)

in the gated CD4⁺ T cells of WT and GCN2KO mice under Th9 induction condition.

Supplementary Fig. 7. The levels of OVA-specific IgE (A) and IgG2b (B) in sera of WT and GCN2KO mice in an OVA-induced AAI model. Data are shown as mean $\pm$ SD, ** $P < 0.01$.

Supplementary Fig. 8. Histological analysis of lung sections of WT and GCN2KO mice using H&E and PAS staining.

Histological score (A) and % of area of PAS positive cells (B) in lung tissues of WT and GCN2KO mice in OVA-induced AAI model. Data are shown as mean $\pm$ SD, ** $P < 0.01$.

Supplementary Fig. 9. Mast cells in lung sections of WT or GCN2KO mice using toluidine blue staining.

Supplementary Fig. 10. Histological analysis of lung sections of RAG2KO mice that received WT or GCN2KO CD4⁺ T cells using H&E and PAS staining.

Histological score (A) and % of area of PAS positive cells (B) in lung tissues of RAG2KO mice that received WT or GCN2KO CD4⁺ T cells in OVA-induced AAI model. Data are shown as mean $\pm$ SD, ** $P < 0.01$.

Supplementary Fig. 11. Mast cells in lung sections of RAG2KO mice that received WT or GCN2KO CD4⁺ T cells using toluidine blue staining.

Supplementary Fig. 12. The levels of OVA-specific IgE in sera of WT mice that received WT-OTII and GCN2KO-OTII Th9 cells in an OVA-induced AAI model. Data are shown as mean $\pm$ SD, ** $P < 0.01$.

Supplementary Fig. 13. Histological analysis of lung sections of WT mice that received WT-OTII or GCN2KO-OTII Th9 cells using H&E and PAS staining.

Histological score (A) and % of area of PAS positive cells (B) in lung tissues of WT mice that received WT-OTII or GCN2KO-OTII Th9 cells in AAI model. Data are shown as mean $\pm$ SD, ** $P < 0.01$.

Supplementary Fig. 14. Mast cells in lung sections of WT mice that received WT-OTII or GCN2KO-OTII Th9 cells using toluidine blue staining.

Supplementary Fig. 15. The percentages of CD4⁺Foxp3⁺ cells in BALF of recipients transferred with WT-OTII and GCN2KO-OTII Th9 cells.

Supplementary Fig. 16. The expressions of *IL-9* in CD4⁺ T cells treated with NF-κB inhibitor CAPE under Th9 induction condition.

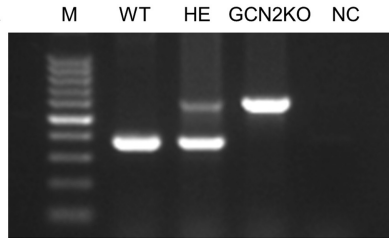
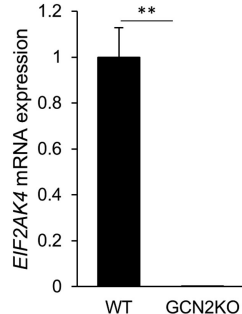
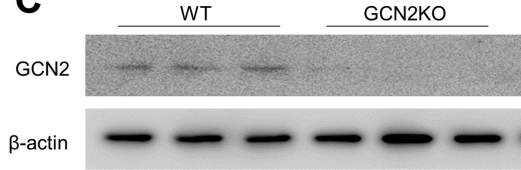
The sorted naïve WT CD4⁺ T cells were cultured under Th9 induction condition. The IL-9 mRNA expression was detected by real-time PCR. Data are shown as mean ± SD, ***P* < 0.01.

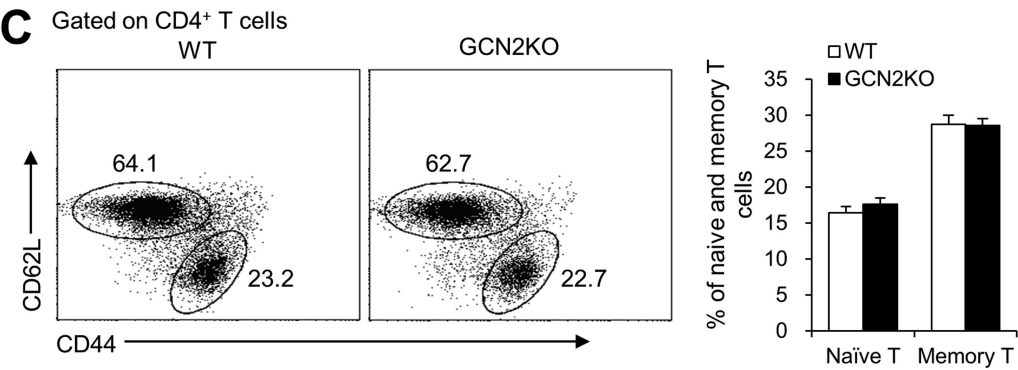
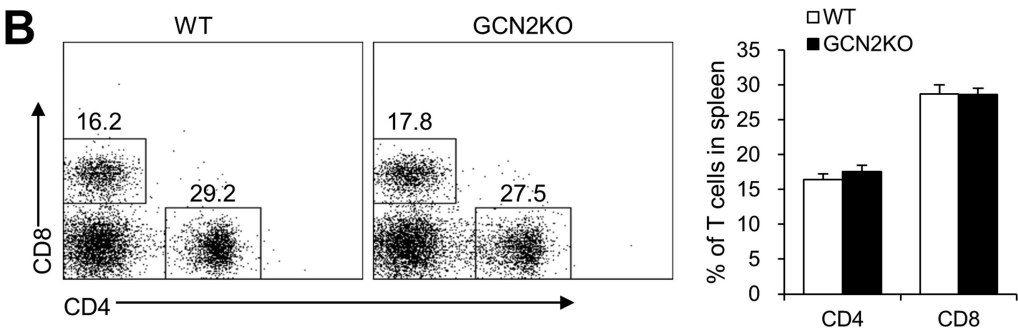
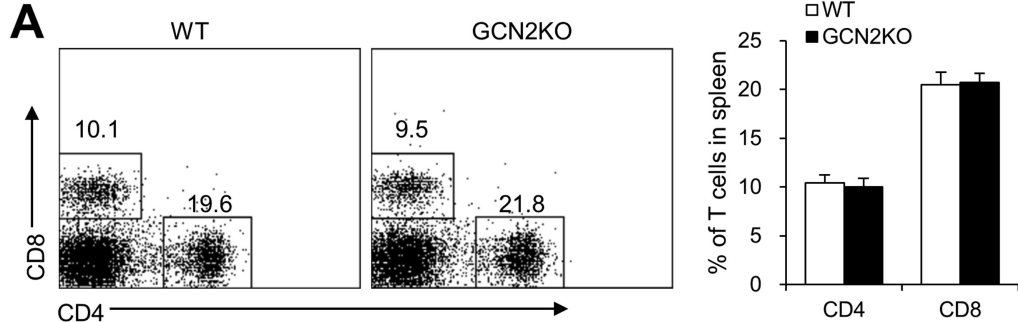
Supplementary Fig. 17. The expressions of Th9-associated molecules and transcription factors.

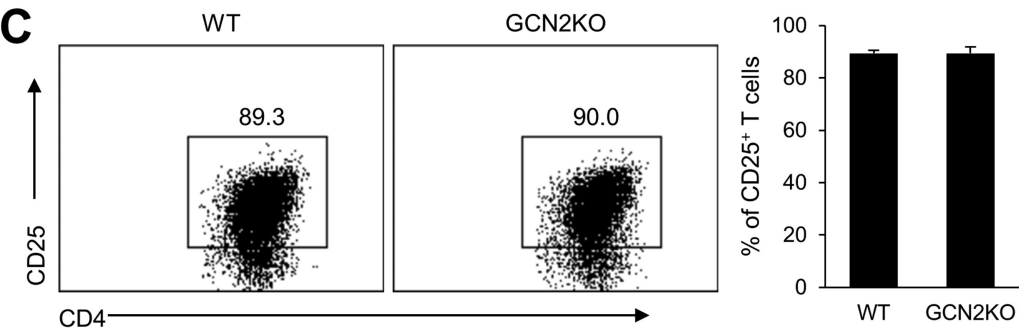
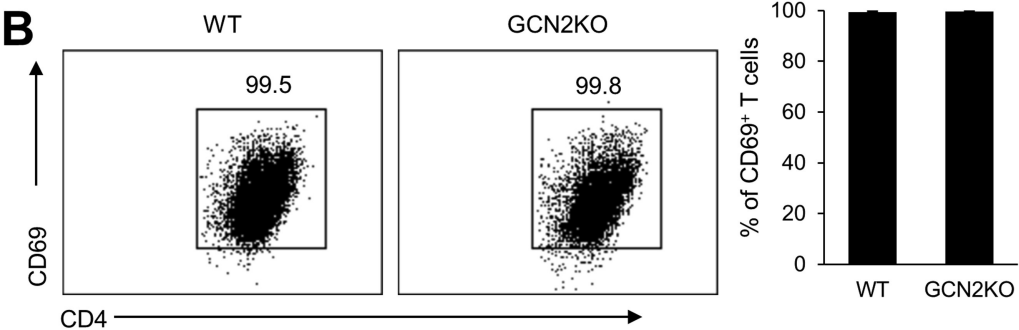
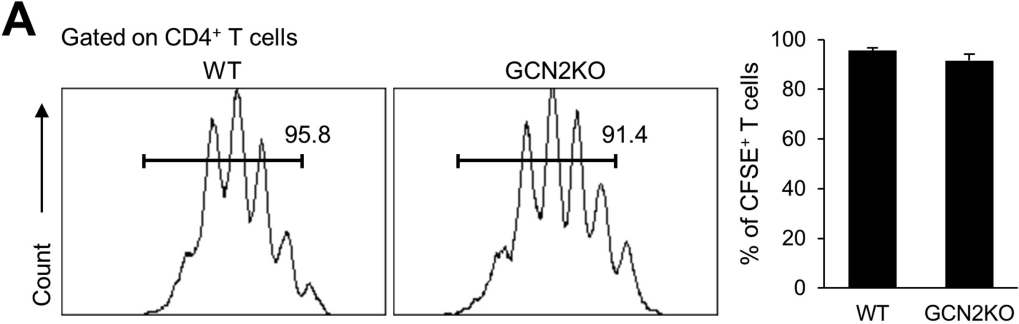
A, Immunoblot analysis of GCN2, p-smad2, Smad2, p-Stat6, Stat6, and β-actin in naïve CD4⁺ T cells of WT and GCN2KO mice under Th9 induction condition for the indicated time points (0-120 mins). **B**, Immunoblot analysis of p-Stat6, Stat6, PU.1, IRFG4, BATF and β-actin in naïve CD4⁺ T cells of WT and GCN2KO mice under Th9 induction condition for 24 h.

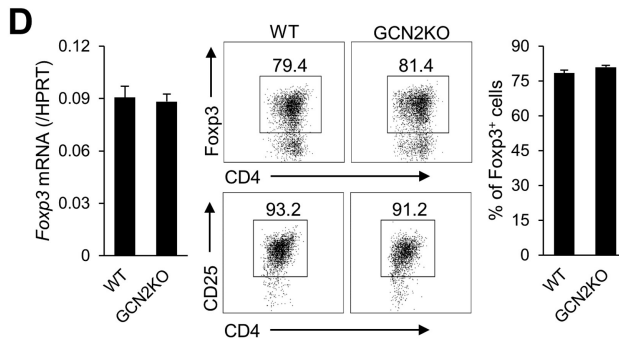
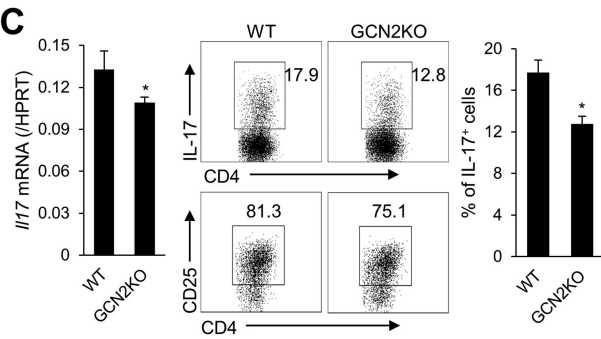
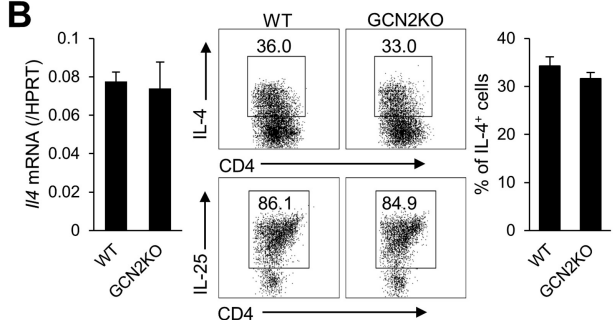
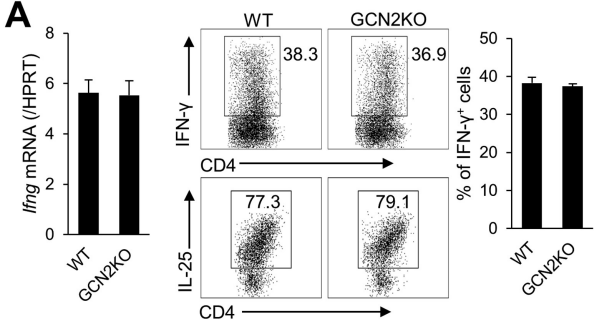
Supplementary Fig. 18. The expressions of *Spi1* and *Irf4* in CD4⁺ T cells stimulated with 2-DG under Th9 induction condition.

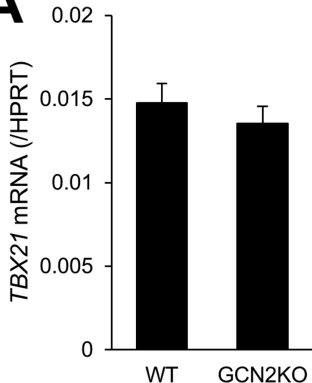
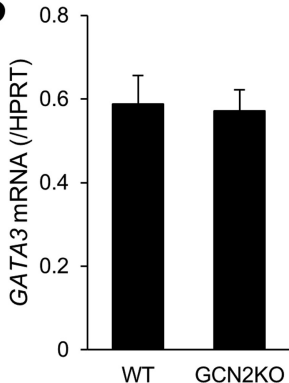
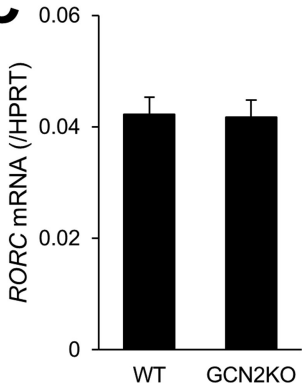
The sorted naïve WT CD4⁺ T cells were cultured under Th9 induction condition. The *Spi1* (**A**) and *Irf4* (**B**) mRNA expressions were detected by real-time PCR. Data are shown as mean ± SD, ***P* < 0.01.

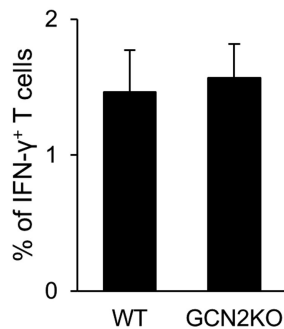
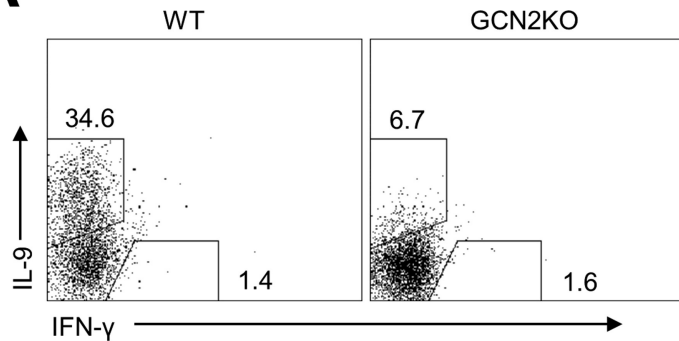
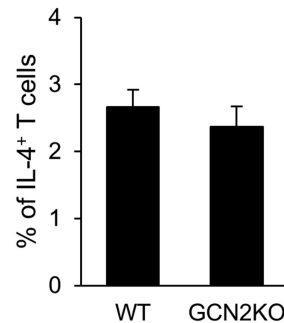
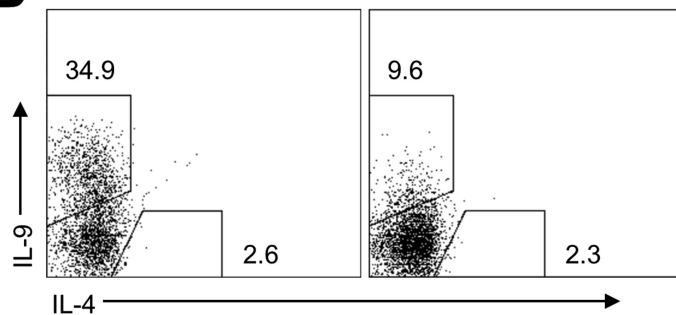
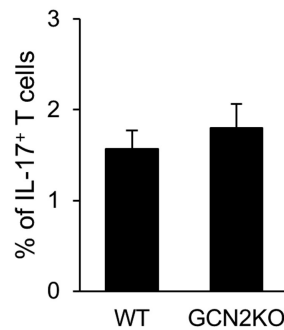
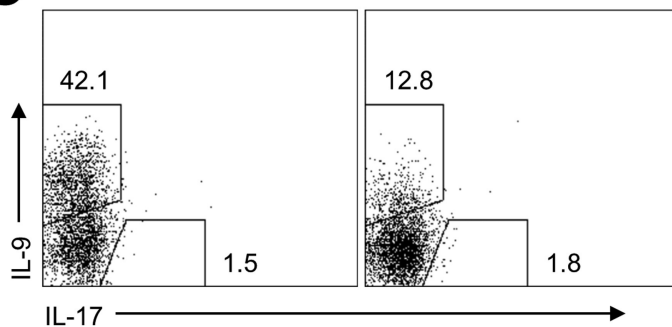
A**B****C**

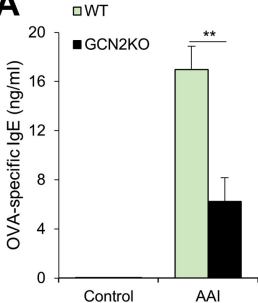
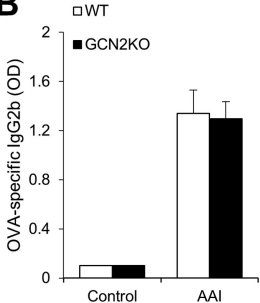


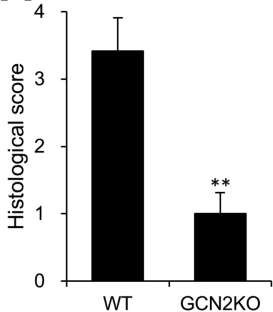
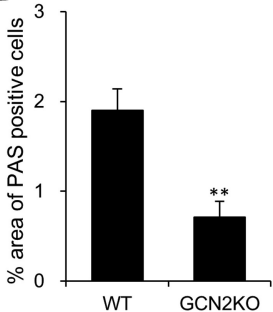




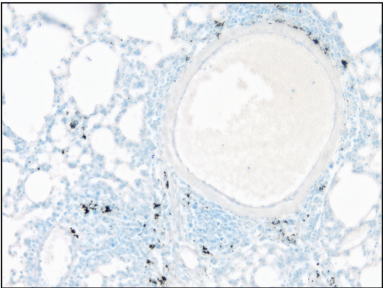
A**B****C**

A Gated on CD4⁺ T cells**B****C**

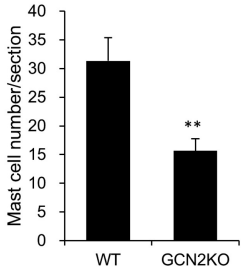
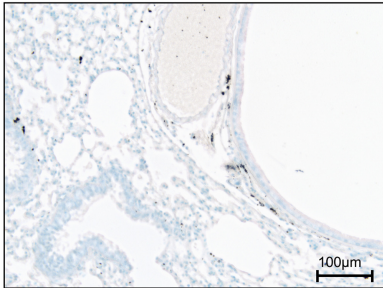
A**B**

A**B**

WT

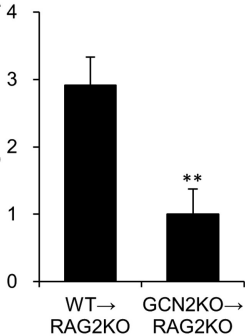


GCN2KO

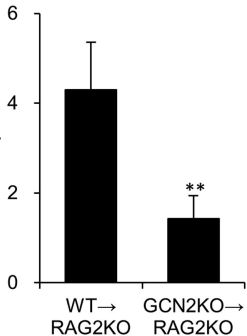


A

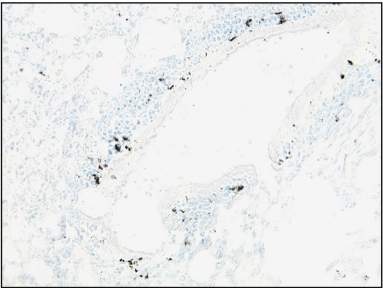
Histological score

**B**

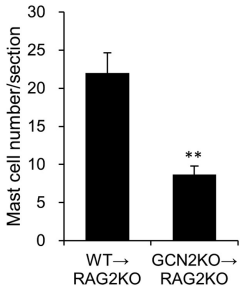
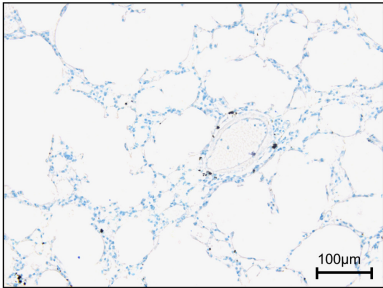
% area of PAS positive cells

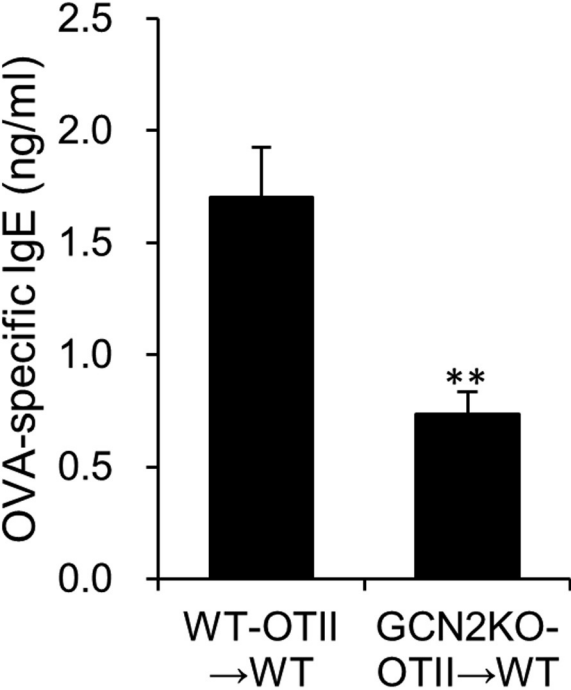


WT→RAG2KO



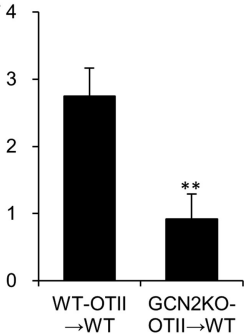
GCN2KO→RAG2KO



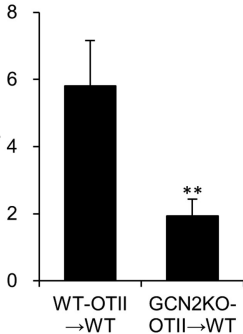


A

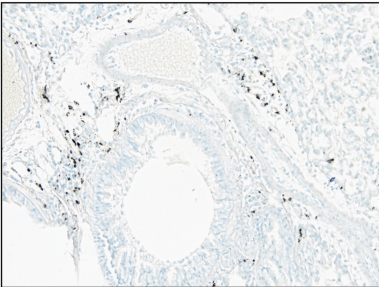
Histological score

**B**

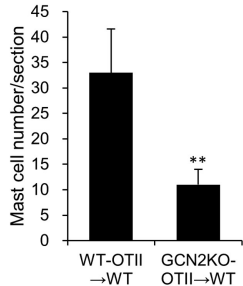
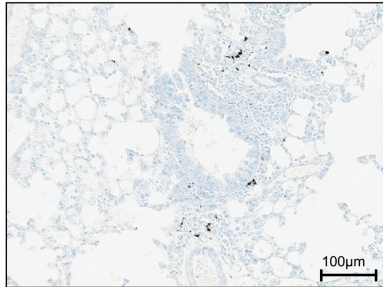
% area of PAS positive cells

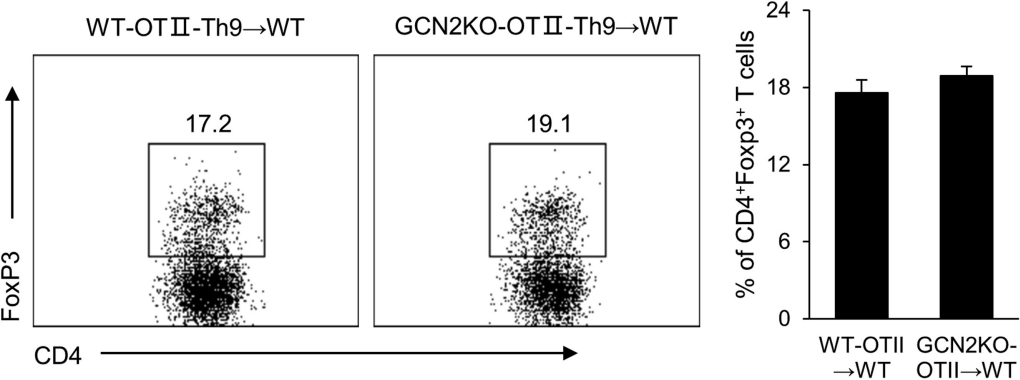


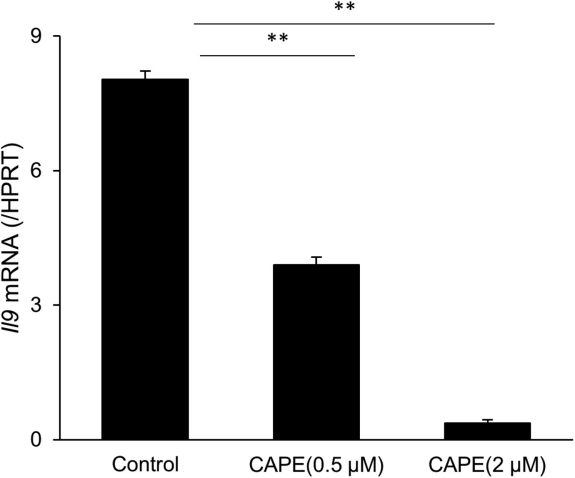
WT-OTII→WT

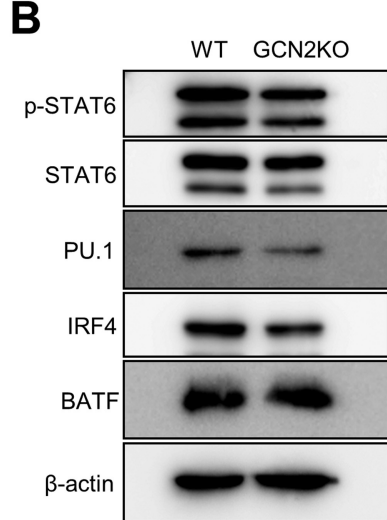
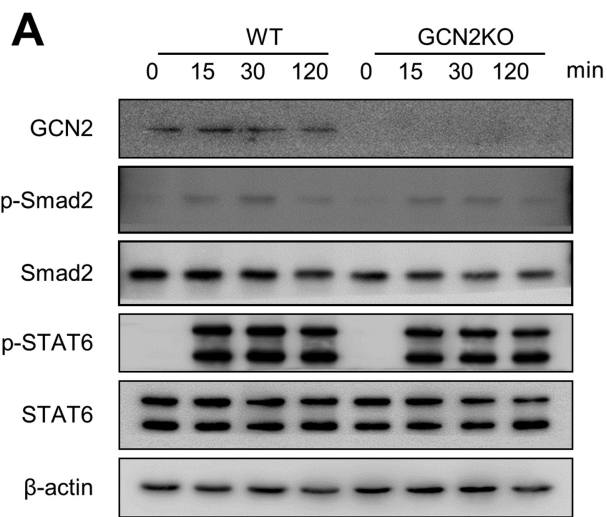


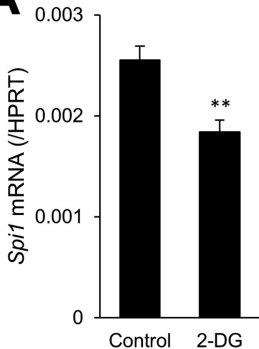
GCN2KO-OTII→WT









A**B**