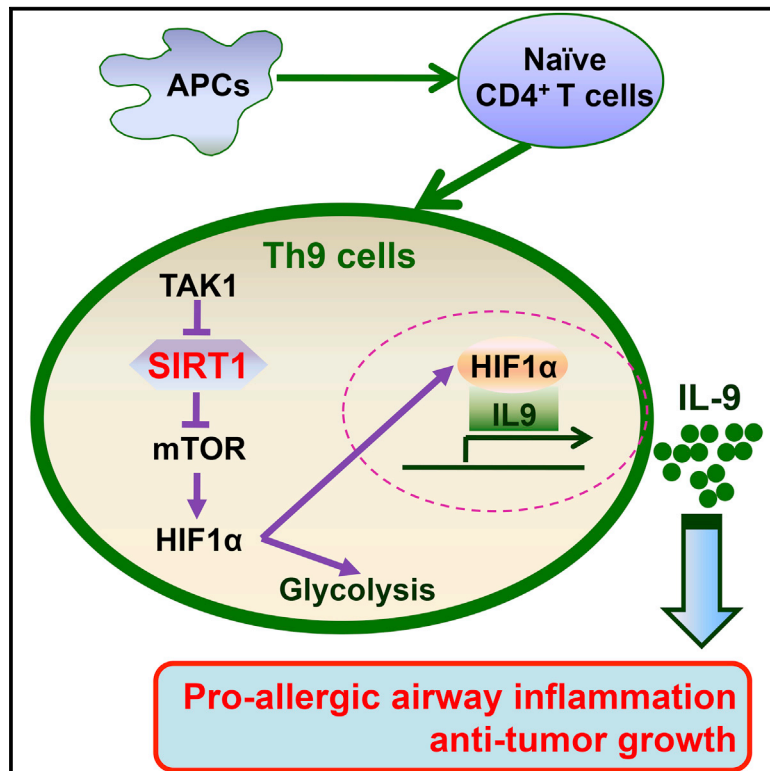


Histone Deacetylase SIRT1 Negatively Regulates the Differentiation of Interleukin-9-Producing CD4⁺ T Cells

Graphical Abstract



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In Brief

Th9 cells are critical in controlling immune-associated diseases. Liu and colleagues demonstrate that histone deacetylase SIRT1 negatively regulates the activities of mTOR and HIF1 α signaling, couples with glycolysis metabolism, suppresses IL-9 production of CD4⁺ T cells, alleviates allergic airway inflammation, and promotes cancerous tumor growth.

Highlights

- Deficiency of SIRT1 in CD4⁺ T cells increased IL-9 production and glycolysis
- Ectopic SIRT1 expression in CD4⁺ T cells inhibited IL-9 production and glycolysis
- SIRT1-dependent Th9 cells regulated tumor and allergic pulmonary inflammation
- TAK1-SIRT1-mTOR-HIF1 α -glycolysis pathway was required for Th9 cell differentiation

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Histone Deacetylase SIRT1 Negatively Regulates the Differentiation of Interleukin-9-Producing CD4⁺ T Cells

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SUMMARY

Distinct metabolic programs support the differentiation of CD4⁺ T cells into separate functional subsets. In this study, we investigated metabolic mechanisms underlying the differentiation of IL-9-producing CD4⁺ T cells (Th9) in allergic airway inflammation and cancerous tumors. We found that histone deacetylase SIRT1 negatively regulated Th9 cell differentiation. A deficiency of SIRT1 induced by either conditional deletion in mouse CD4⁺ T cells or the use of small interfering RNA (siRNA) in mouse or human T cells increased IL-9 production, whereas ectopic SIRT1 expression inhibited it. Notably, SIRT1 inhibited Th9 cell differentiation that regulated anti-tumor immunity and allergic pulmonary inflammation. Glycolytic activation through the mTOR-hypoxia-inducible factor-1 α (HIF1 α) was required for the differentiation of Th9 cells that conferred protection against tumors and is involved in allergic airway inflammation. Our results define the essential features of SIRT1-mTOR-HIF1 α signaling-coupled glycolytic pathway in inducing Th9 cell differentiation, with implications for metabolic reprogramming as an immunotherapeutic approach.

INTRODUCTION

The engagement of a variety of specific metabolic pathways profoundly affects T cell differentiation and function (Man and Kalies, 2015). Therefore, it is no surprise that distinct metabolic programs also support the differentiation of CD4⁺ T helper (Th) cells into their separate functional subsets (Zeng and Chi, 2015). It has been demonstrated that Th1, Th2, and Th17 cells are strongly dependent on glycolysis, whereas regulatory T (Treg) cells depend more on the oxidation of lipids (Waickman and Powell, 2012). However, the specific modulation of metabolism that is involved in Th9 cell differentiation remains unclear.

SIRT1 is the highly conserved mammalian NAD⁺-dependent histone deacetylase and has emerged as a key metabolic sensor in various metabolic tissues (Yang et al., 2015). In response to different environmental stimuli, SIRT1 directly links to the cellular metabolism of gene expression, thereby modulating a variety of cellular processes, such as energy metabolism and the stress response (Lim et al., 2010; Yang et al., 2015). SIRT1 is also a key transcriptional regulator of inflammation. In innate immune cells, SIRT1 inhibits the production of proinflammatory cytokines in macrophages and dendritic cells (DCs) (Yang et al., 2015). In myeloid-derived suppressor cells (MDSCs), SIRT1 directs the differentiation of MDSCs in anti-tumor immunity (Liu et al., 2014). Furthermore, DC SIRT1 has been reported to direct T cell differentiation from Th1 to Treg cells in the course of microbial-associated infection (Liu et al., 2015) and to promote Th2 cell differentiation in airway allergy (Legutko et al., 2011). In addition, SIRT1 is required to maintain T cell tolerance (Yang et al., 2015). However, whether SIRT1 is involved in the metabolism-dependent modulation of the differentiation of intrinsic T cells remains unclear.

In the present study, we have demonstrated that SIRT1-dependent glycolytic metabolism modulation is critical for directing the differentiation of Th9 cells under physiological conditions as well as pathological conditions such as Th9 cell-associated allergic airway inflammation and tumors. Directly targeting the *Il9* gene locus, SIRT1 inhibits the differentiation of Th9 cells by an mTOR-HIF1 α -dependent signaling coupled with glycolytic pathway.

RESULTS

Th9 Cell Differentiation Is Associated with SIRT1-Dependent Glycolytic Activity

Although metabolic signaling regulates T cell differentiation, the mechanism of glycolytic metabolism in different T cell subsets remains unclear. We therefore investigated the glycolytic activities in various T cell subsets. As previously reported (Liu et al., 2010), Th1, Th2, Th17, Treg, and Th9 cells could be induced in vitro and obtained (Figure 1A, left). The glycolytic activity of each T cell subset was measured by the generation of ³H-labeled H₂O from [3-³H]-glycose. Consistent with a previous report, Th1,

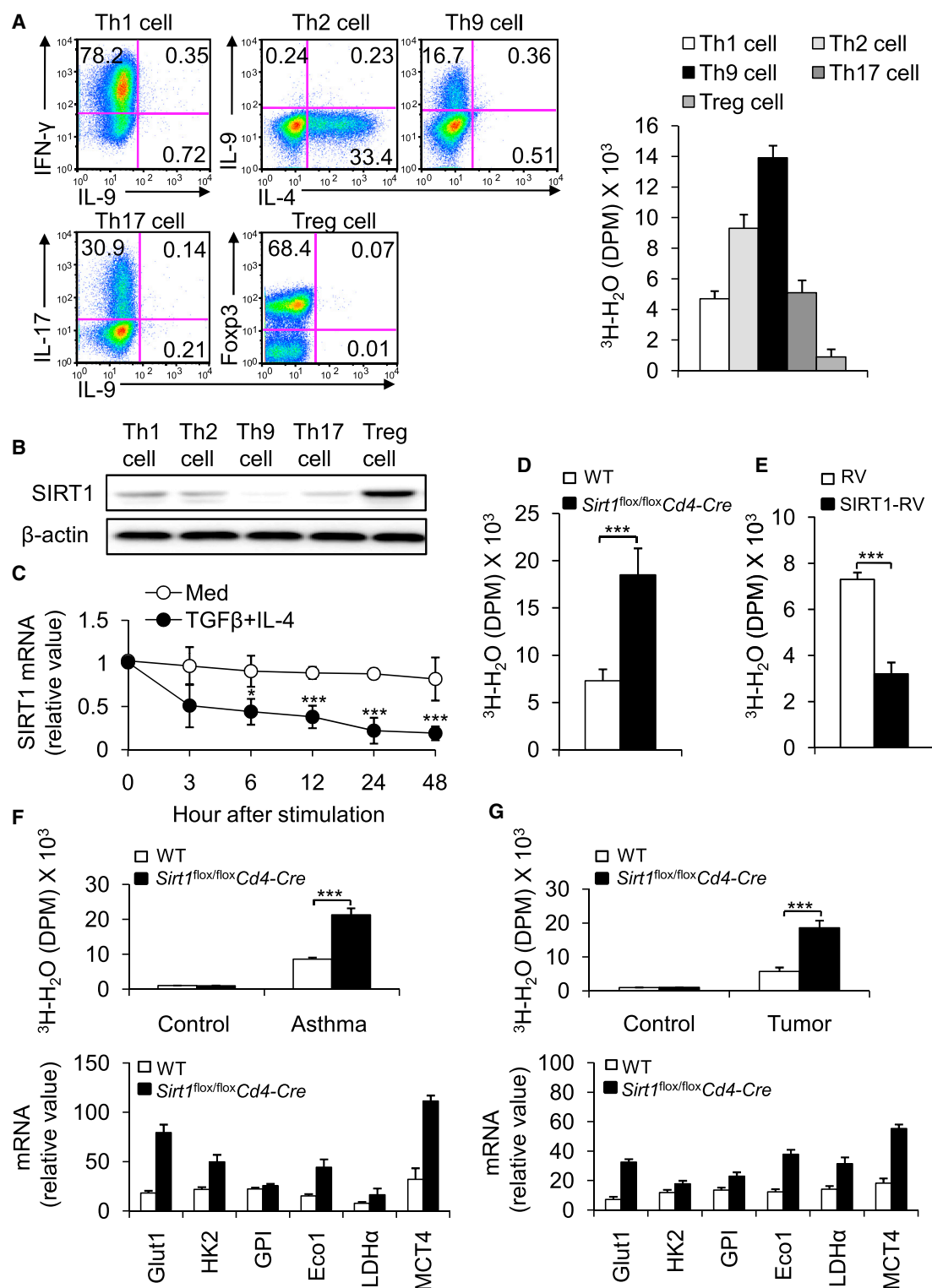


Figure 1. The Th9 Cell-Inducing Condition and Th9 Cell-Associated Allergic Airway Inflammation and Tumors along with Alterations in SIRT1-Dependent Glycolytic Activity

(A) Sorted naive T cells ($\text{CD4}^+\text{TCR}^+\text{CD62L}^{\text{hi}}\text{CD44}^{\text{lo}}\text{CD25}^-$) from WT mice were differentiated under Th1, Th2, Th9, Th17, or Treg cell-inducing conditions for 5 days, followed by intracellular staining of IFN- γ (Th1 cells), IL-4 (Th2 cells), IL-17 (Th17 cells) or Foxp3 (Treg cells), and IL-9 (left). The glycolytic activity of these cells was measured by the generation of ^3H -labeled H_2O from [^3H]-glycose (right).

(B) SIRT1 expression was examined by western blot in T cells activated under various conditions for 5 days.

(legend continued on next page)

Th2, and Th17 cells exhibited much higher while Treg cells exhibited less glycolytic activity (Shi et al., 2011). Moreover, Th9 cells exhibited more glycolytic activity than Th1, Th2, Th17, or Treg cells (Figure 1A, right). These results suggest that Th9 cell differentiation is probably related to glycolytic metabolism.

The key metabolic regulator SIRT1 that has previously been shown to target dendritic cells (DCs) regulates the differentiation of Th1 and Treg cells (Liu et al., 2015). We investigated whether SIRT1 regulates the glycolytic activity of T cell subsets. SIRT1 was found to be differentially induced in all of the T cell subsets (Figures 1B and S1A). Treg cells exhibited the highest expression, whereas Th9 cells exhibited a lower expression than Th1, Th2, and Th17 cells (Figure 1B). Consistent with this result, a Th9 cell-inducing condition (IL-4 plus TGF- β 1) resulted in a decrease of SIRT1 mRNA (Figures 1C and S1A). IL-4 alone did not affect the expression of SIRT1, but TGF- β 1 plus IL-4 caused the most significant downregulation of SIRT1 (Figure S1B). This result suggests that the optimized Th9 cell-polarizing cytokine milieu is critical for suppressing SIRT1. We next studied the molecular mechanisms underlying the TGF- β 1-mediated downregulation of SIRT1 in Th9 cells. We found that Smad3, an inhibitor of transcriptional factor Smad3, an important mediator of a canonical TGF- β 1 signaling, did not alter the SIRT1 expression, although it did suppress the expression of IL-9 (Figures S1C and S1D). This result suggested that the Smad-dependent pathway is not involved in regulating SIRT1 in Th9 cell differentiation. The TGF- β -triggered kinase TAK1 is an important mediator of the TGF- β signaling that is independent of Smad (Choi et al., 2012). TAK1 has also been reported as a key player in directing Th9 cell differentiation (Nakatsukasa et al., 2015). We therefore investigated the role of TAK1 in regulating SIRT1 expression during Th9 cell differentiation. We found that 5z-7-oxozeanin, a potent TAK1 inhibitor, completely blocked IL-9 expression but did not substantially change the differentiation of Th1, Th2, Th17, or Treg cells (Figure S1E). These data confirmed the critical role of TAK1 in Th9 cell differentiation as previously reported. Importantly, the inhibition of TAK1 significantly reversed the suppression of SIRT1 (Figure S1F), which suggested that Smad-independent TAK1 signal was responsible for the suppression of SIRT1 during Th9 cell differentiation. Consistent with the pharmacologic result, the TAK1 genetic silencing suppressed IL-9 production and reversed the downregulation of SIRT1 expression during Th9 cell differentiation (Figures S1G–S1I). Together, these data suggest that the TAK1 selectively suppresses SIRT1 during Th9 cell differentiation.

To test the role of SIRT1 in Th9 cell differentiation, we crossed *Sirt1*^{flox/flox} and *Cd4-Cre* mice to conditionally delete *Sirt1* gene expression in T cells (called *Sirt1*^{flox/flox}*Cd4-Cre* mice hereafter). Immunoblot and real-time PCR results showed that SIRT1 was specifically deleted in T cells in this mouse (Figures S1J and S1K). Thus, we observed the glycolytic activity of Th9 cells obtained from *Sirt1*^{flox/flox}*Cd4-Cre* mice. Sorted naive T cells (CD4⁺TCR⁺CD62L^{hi}CD44^{lo}CD25[−] cells) from both the wild-type (WT) and *Sirt1*^{flox/flox}*Cd4-Cre* mice were differentiated under Th9 or other T cell subset-inducing conditions, and the glycolytic activity was measured as described above. SIRT1 deficiency increased the glycolytic activity in Th9 cells (Figures 1D and S2A). This suggested that SIRT1 is required for the glycolytic modulation of Th9 cells. To test whether SIRT1 is sufficient for the glycolytic activity of Th9 or other T cell subsets, we ectopically expressed SIRT1 with retrovirus under various T cell subset-inducing conditions. Modest SIRT1 overexpression was shown to inhibit Th9 cell glycolytic activity (Figures 1E and S2B). Thus, these data demonstrate that SIRT1 is involved in the glycolytic modulation of Th9 cell differentiation.

To ascertain the role of SIRT1-dependent glycolytic activity in regulating Th9 cell differentiation, we directly compared Th9 cells from Th9-associated diseases. We subjected WT and *Sirt1*^{flox/flox}*Cd4-Cre* mice to a standard protocol for the development of ovalbumin (OVA)-induced allergic airway inflammation in which Th9 cells have been shown to be involved (Wilhelm et al., 2011). We sensitized mice with OVA in aluminum hydroxide and challenged them intranasally with OVA in PBS. CD4⁺ T cells were isolated from lung-local draining lymph nodes (DLNs). As expected, there were markedly reduced SIRT1 mRNA and increased IL-9 mRNA expression in OVA-sensitized asthma compared with control cells (Figures S2C and S2D). These results indicate that SIRT1 downregulation is probably negatively related in IL-9-dependent allergic airway inflammation. Furthermore, the glycolytic activity of T cells was measured in both WT and *Sirt1*^{flox/flox}*Cd4-Cre* mice in OVA-sensitized asthma. *Sirt1*^{flox/flox}*Cd4-Cre* mice exhibited much more glycolytic activity than WT mice (Figure 1F, top). These findings suggest that SIRT1-dependent glycolytic activity is associated with Th9 cell differentiation in allergic airway inflammation.

Recently, Th9 cells have been reported to inhibit tumor growth (Purwar et al., 2012). To directly observe the role of Th9 cells in a tumor model, we used a T cell adoptive transfer system. Isolated naive T cells from WT or *Sirt1*^{flox/flox}*Cd4-Cre* mice were differentiated under Th9 cell-inducing conditions and the same number

(C) SIRT1 mRNA expression as examined by real-time PCR analysis in T cells activated by Th9 cell-inducing conditions for different time courses. Expressions in naive cells were set to 1.

(D) Naive T cells from WT and *Sirt1*^{flox/flox}*Cd4-Cre* mice were differentiated under Th9 cell conditions, followed by the measurement of glycolytic activity.

(E) eGFP⁺ cells sorted from the Th9 cell condition that were transduced with control retrovirus (RV) or *Sirt1*-expressing retrovirus (SIRT1-RV) were measured for glycolytic activity.

(F) WT and *Sirt1*^{flox/flox}*Cd4-Cre* mice were sensitized and challenged intranasally with OVA; T cells were isolated from pulmonary DLNs at 48 hr after the final intranasal challenge. The glycolytic activity of these cells was measured by the generation of ³H-labeled H₂O from [3-³H]-glucose (top). RNA was isolated from DLN T cells in WT or *Sirt1*^{flox/flox}*Cd4-Cre* mice and stimulated for 72 hr with anti-CD3, then used for RT-PCR analysis of glycolytic molecules (bottom).

(G) *Rag1*^{−/−} mice were given intravenous transfer of WT or *Sirt1*^{flox/flox}*Cd4-Cre* naive CD4⁺ T cells (stimulated via anti-CD3 and anti-CD28, together with TGF- β 1 and IL-4 for 3 days) along with simultaneous subcutaneous injection of B16 melanoma cells on day 0. After 30 days, CD4⁺ T cells were isolated from mouse solid tumors in WT or *Sirt1*^{flox/flox}*Cd4-Cre* mice and the glycolytic activity of these cells was measured by the generation of ³H-labeled H₂O from [3-³H]-glycose. All tumor tissues were subjected to the same enzymatic digestion (top). RNA was isolated from tumor-derived T cells in WT or *Sirt1*^{flox/flox}*Cd4-Cre* mice and these cells were stimulated for 72 hr with anti-CD3, and then used for RT-PCR analysis of glycolytic molecules (bottom).

Data are representative of three to four independent experiments (n = 4). *p < 0.05; ***p < 0.001, compared with the indicated groups. See also Figures S1 and S3.

of cells intravenously injected into *Rag1*^{-/-} mice, which have a congenital deficiency in mature B cells and T cells. Then we subcutaneously injected B16 melanoma cells into the *Rag1*^{-/-} recipient mice. On day 20 the mice received CD4⁺ T cells, the tumor tissue was digested, and the CD4⁺ T cells were isolated. As expected, SIRT1 mRNA expression was markedly reduced whereas IL-9 mRNA expression in tumors was increased compared with control cells (Figures S2E and S2F). These data suggest that SIRT1 downregulation is negatively related to IL-9 production in tumors. Furthermore, *Sirt1*^{flox/flox}*Cd4-Cre* T cells exhibited much more glycolytic activity than WT control (Figure 1G, top). This finding indicates that SIRT1-dependent glycolytic activity is probably associated with Th9 cell differentiation in tumors.

There are multiple glucose-associated enzymes that play an important role in controlling the generation of lactate and ATP molecules as energy sources. Consistent with this, transcriptional mRNA analyses of Glut1, glucose-6-phosphate isomerase, Eno1 (enolase 1), LDH α , and monocarboxylic acid transporter member 4 in WT T cells isolated from lung DLNs in asthma (Figure 1F, bottom) or a tumor (Figure 1G, bottom) revealed marked upregulation of genes encoding various molecules involved in glycolysis. Taken together, the SIRT1-dependent glycolytic activities associated with Th9 cell differentiation.

SIRT1 Inhibits Th9 Cell Differentiation

To clarify the role of SIRT1 in Th9 cell differentiation, we ectopically expressed SIRT1 with a retrovirus and sorted the positive green fluorescent protein (GFP) T cells, observing the alteration in IL-9 expression that took place under various T cell differentiation-inducing conditions, as described above. Modest SIRT1 overexpression (SIRT1-RV) resulted in a decrease in IL-9 mRNA expression, IL-9 secretion, and the ratio of IL-9⁺CD4⁺ T cells under Th9 cell-inducing conditions, but not Th1, Th2, Th17, or Treg cell-inducing conditions, compared with control (RV; Figures 2A–2C). In addition, even IL-4 expression was not altered in the SIRT1-RV group under either Th0 or Th9 cell-inducing conditions (Figure 2C). Moreover, IL-9 mRNA expression and IL-9 secretion exhibited potent and sustained downregulation in SIRT1-RV compared with RV (Figures 2D and 2E). Importantly, consistent with the alteration in glycolytic activity (Figure 1E), the glucose-associated enzyme molecules were diminished in SIRT1-RV compared with RV (Figure 2F), indicating that the SIRT1-dependent glycolytic is sufficient to regulate Th9 cell differentiation.

SIRT1 Deficiency Enhances Th9 Cell Differentiation

To ascertain the role of SIRT1 in Th9 cell differentiation, we obtained differentiated naive T cells from WT and *Sirt1*^{flox/flox}*Cd4-Cre* mice under various T cell subset-inducing conditions, as described above. We found that IL-9 mRNA expression, IL-9 secretion, and the IL-9⁺ cell ratio in T cells from *Sirt1*^{flox/flox}*Cd4-Cre* were higher than the WT under Th9 cell-inducing conditions (Figures 3A–3C). Naive WT CD4⁺ T cells did not produce IL-9 after being cultured with either TGF- β 1 or IL-4 alone. However, naive *Sirt1*^{flox/flox}*Cd4-Cre* T cells readily produced a substantial amount of IL-9 in response to either TGF- β 1 or TGF- β 1 plus IL-4 under Th9 cell-inducing conditions in an IL-4 dose-dependent manner (Figures 3C and S2G). Moreover, IL-9

mRNA expression and IL-9 secretion exhibited strong and sustained upregulation in *Sirt1*^{flox/flox}*Cd4-Cre* compared with the WT (Figures 3D and 3E). Importantly, consistent with the glycolytic activity (Figure 1D), the glucose-associated enzyme molecules were significantly increased in *Sirt1*^{flox/flox}*Cd4-Cre* compared with the WT (Figures S2H and S2I). Notably, we also acutely deleted SIRT1 in naive WT CD4⁺ T cells using siRNA. SIRT1 efficient silencing resulted in the upregulation of IL-9 mRNA expression and a much higher IL-9⁺ cell ratio in CD4⁺ T cells compared with control (Figures 3F and 3G). Consistent with this, the glycolytic activity was significantly increased in SIRT1 siRNA compared with control cells (Figure S2J), indicating that SIRT1-dependent glycolytic activity is required for directing Th9 cell differentiation.

To understand how SIRT1 regulates Th9 cell differentiation, we compared the gene expression profiles of WT and *Sirt1*^{flox/flox}*Cd4-Cre* cells differentiated toward Th9 cells using Affymetrix oligonucleotide arrays and found that the expression of a selective groups of metabolic molecules, including glycolytic enzymes described in Figures 1F and 1G, were upregulated in *Sirt1*^{flox/flox}*Cd4-Cre* cells. *Sirt1*^{flox/flox}*Cd4-Cre* cells also displayed altered expression patterns of transcription factors, signaling molecules, and surface or secreted factors, including Hif1 α , and, as expected, IL-9 (Figure 3H). These data demonstrate that diverse SIRT1 target genes in T cells regulate T cell differentiation and metabolism.

The IL-9 Produced by SIRT1 Deficiency CD4⁺ T Cells Is Involved in the Development of Allergic Airway Inflammation

It has been reported that IL-9 is involved in allergic airway inflammation (Wilhelm et al., 2011). To investigate the role of SIRT1-dependent Th9 cell differentiation, we set up an OVA-induced allergic airway inflammation model in WT and *Sirt1*^{flox/flox}*Cd4-Cre* mice, as described above. *Sirt1*^{flox/flox}*Cd4-Cre* mice exhibited significantly more severely pathogenic lung inflammation and greater inflammatory cell recruitment, including eosinophil infiltration in bronchoalveolar lavage fluid (BALF), compared with the WT (Figures 4A, 4B, S3A, and S3B). The CD4⁺ T cells isolated from the lung DLNs of *Sirt1*^{flox/flox}*Cd4-Cre* model mice exhibited a greater number of IL-9⁺ cells, but not IFN- γ ⁺, IL-4⁺, IL-17⁺, or Foxp3⁺ cells, compared with WT (Figures 4C, S3C, and S3D). Moreover, SIRT1 deficiency resulted in higher IL-9 secretion (Figure 4D) accompanied by higher glycolytic activity (Figure S3E). The proliferation of antigen-specific T cells, however, was similar (Figure S3F). Importantly, blocking IL-9 with an anti-IL-9 antibody markedly restored all these pathological alterations (Figures 4A–4D and S3A–S3D). Taken together, the data show that SIRT1 is required for suppressing Th9 cell differentiation in alleviating Th9 cell-associated allergic airway inflammation.

To directly test the role of SIRT1 in Th9 cell-dependent diseases, we used an adoptive transfer model. Naive T cells isolated from WT or *Sirt1*^{flox/flox}*Cd4-Cre* mice were differentiated under Th9 cell-inducing conditions and transferred into *Rag1*^{-/-} mice, which were sensitized and challenged with OVA on day 0. The mice that received *Sirt1*^{flox/flox}*Cd4-Cre* CD4⁺ T cells displayed severe pathological lung damage and eosinophil infiltration in BALF compared with the WT (Figures S4A and S4B).

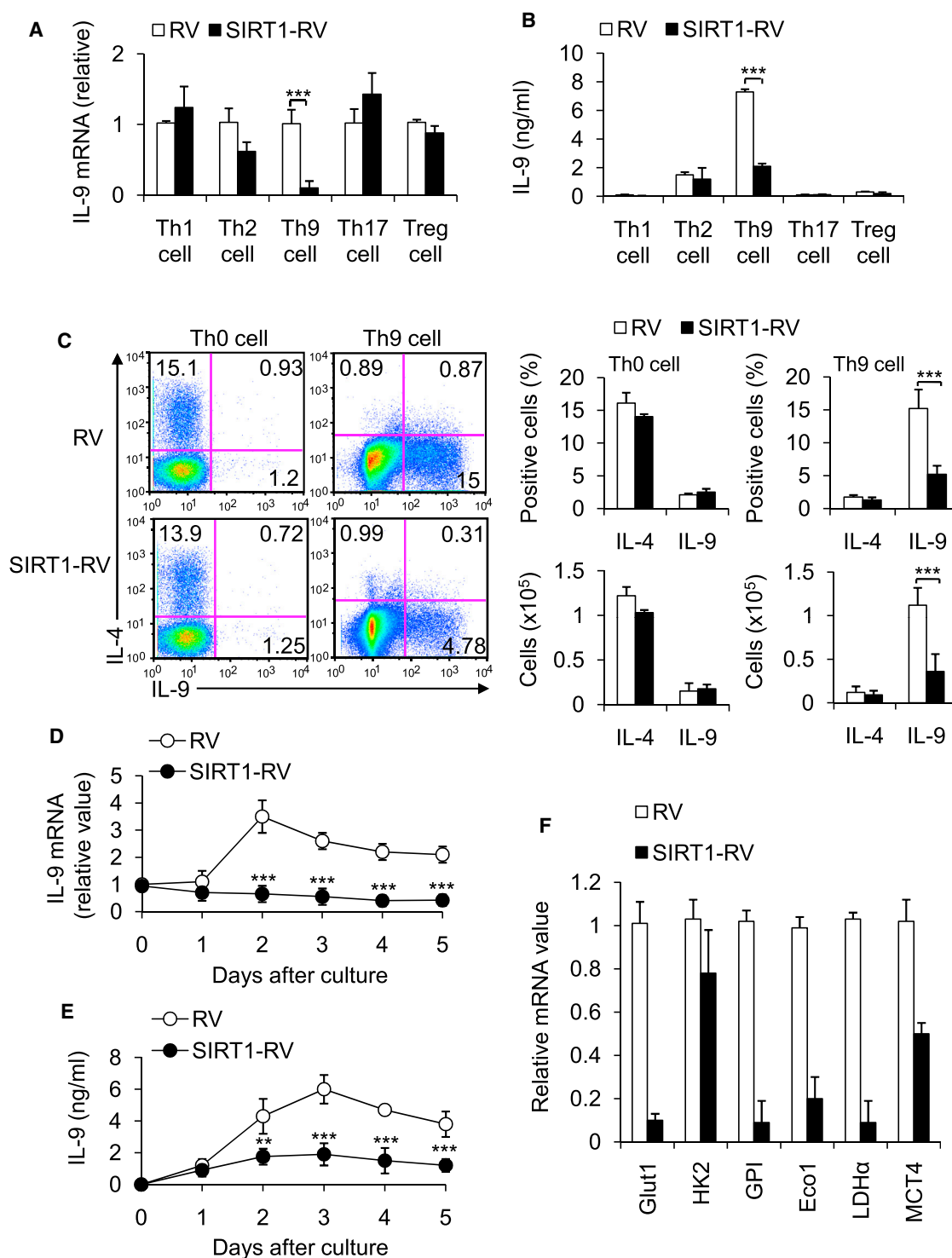


Figure 2. SIRT1 Inhibits Th9 Cell Differentiation

(A and B) IL-9 expression (A) and IL-9 secretion (B) by eGFP⁺ cells sorted from the indicated cell-inducing conditions were transduced with control retrovirus (RV) or SIRT1-expressing retrovirus (SIRT1-RV) and stimulated for 24 hr with anti-CD3. Expressions in the RV groups were set to 1.

(C) Intracellular staining of IL-4 and IL-9 in the eGFP⁺ populations from the Th9 or Th0 cell-inducing conditions transduced with RV or SIRT1-RV.

(D and E) The time course changes in IL-9 mRNA expression (D) and IL-9 secretion (E) by eGFP⁺ cells from the Th9 cell conditions transduced with RV or SIRT1-RV. (F) Glycolytic molecule expressions by eGFP⁺ cells sorted from the Th9 cell-inducing conditions transduced with RV or SIRT1-RV and stimulated for 24 hr with anti-CD3, then used for RT-PCR analyses of glycolytic molecules (expressions in the RV groups were set to 1).

Data are representative of three to four independent experiments (n = 4). **p < 0.01; ***p < 0.001, compared with the indicated groups.

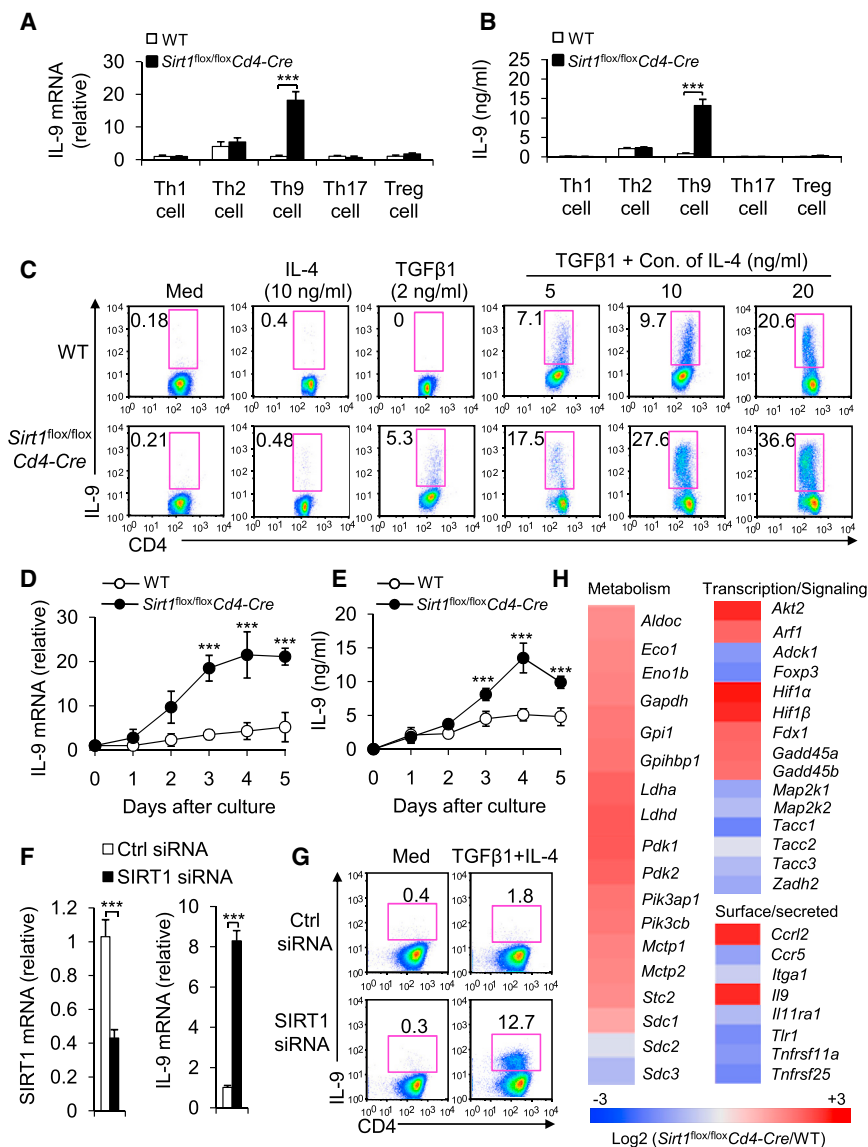


Figure 3. SIRT1 Deficiency Enhances Th9 Cell Differentiation

(A and B) IL-9 expression (A) and IL-9 secretion (B) by CD4⁺ T cells sorted from the indicated cell-inducing conditions in WT or *Sirt1*^{fllox/fllox} *Cd4-Cre* mice and stimulated for 24 hr with anti-CD3. Expressions in WT naive T cells were set to 1.

(C) Intracellular staining of IL-9 in WT and *Sirt1*^{fllox/fllox} *Cd4-Cre* CD4⁺ T cells cultured for 72 hr with anti-CD3 and anti-CD28, plus medium alone or the various indicated combinations.

(D and E) The time course changes in IL-9 expression (D) and secretion (E) by CD4⁺ cells under Th9 cell-inducing conditions in WT and *Sirt1*^{fllox/fllox} *Cd4-Cre* mice. Expressions in WT naive T cells were set to 1.

(F) Expression of SIRT1 (left) and IL-9 mRNA (right) in naive CD4⁺ T cells from WT mice transfected with control siRNA (Ctrl siRNA), or SIRT1-specific siRNA (SIRT1 siRNA), and stimulated for 48 hr with anti-CD3 and anti-CD28 plus TGF-β1 and IL-4 (expressions in Ctrl siRNA were set to 1).

(G) Intracellular IL-9 expression in cells transfected as in (F) and stimulated with anti-CD3 and anti-CD28 plus medium (med) alone or TGF-β1 and IL-4.

(H) Naive T cells from WT and *Sirt1*^{fllox/fllox} *Cd4-Cre* mice were differentiated under Th9 cell-inducing condition for 3 days, and then RNA was analyzed by microarrays to compare the expression profiles of the WT and *Sirt1*^{fllox/fllox} *Cd4-Cre* cells with certain genes involved in metabolism, transcription factors, or signaling pathways, and surface or secreted molecules. Heat maps display gene expression relative to the WT mean on a log2 scale.

Data are representative of three to four independent experiments (A–G; n = 3–5). ***p < 0.001, compared with the indicated groups. See also Figures S1 and S3.

Infiltrating CD4⁺ T cells isolated from the lung DLNs of the *Sirt1*^{fllox/fllox} *Cd4-Cre* group were stimulated with anti-CD3 for 24 hr and exhibited higher IL-9 expression and glycolytic activity compared with the control (Figures S4C and S4D). However, blocking IL-9 restored all these pathological alterations (Figure S4). Thus, these data indicate that SIRT1 is required for Th9 cell-dependent allergic airway inflammation.

The IL-9 Produced by SIRT1-Deficient CD4⁺ T Cells Is Involved in Delaying Tumor Growth

Recently, Th9 cells have been reported to inhibit tumor growth (Purwar et al., 2012). To directly investigate the role of SIRT1-dependent Th9 cell differentiation, we set up a tumor model in WT and *Sirt1*^{fllox/fllox} *Cd4-Cre* mice, as described above. Naive T cells isolated from WT or *Sirt1*^{fllox/fllox} *Cd4-Cre* mice were differentiated under Th9 cell-inducing conditions and transferred into *Rag1*^{−/−} mice, and then we subcutaneously injected these mice with B16 melanoma cells. The mice that received

Sirt1^{fllox/fllox} *Cd4-Cre* CD4⁺ T cells exhibited tumors of smaller size compared with the WT control (Figure 4E). Tumor-infiltrating CD4⁺ T cells isolated from the *Sirt1*^{fllox/fllox} *Cd4-Cre* groups displayed higher IL-9 expression, IL-9 secretion, and IL-9⁺ ratio accompanied by higher glycolytic activity, compared with WT (Figures 4F–4H and S5). Importantly, blocking IL-9 with an anti-IL-9 antibody restored the *Sirt1*^{fllox/fllox} *Cd4-Cre* Th9 cell transfer-elicited changes, except for the glycolytic activity (Figures 4E–4H and S5). Taken together, these data show that SIRT1 is required for suppressing Th9 cell differentiation in inhibiting Th9 cell-dependent anti-tumor immunity.

Blocking the Glycolytic Metabolism Pathway Restores SIRT1-Deficiency-Induced Th9 Cell Differentiation

To investigate the role of SIRT1-dependent glycolytic activity in Th9 cell differentiation, naive T cells were differentiated under Th9 cell-inducing conditions in the presence or absence of 2-DG, a prototypical inhibitor of the glycolytic pathway that

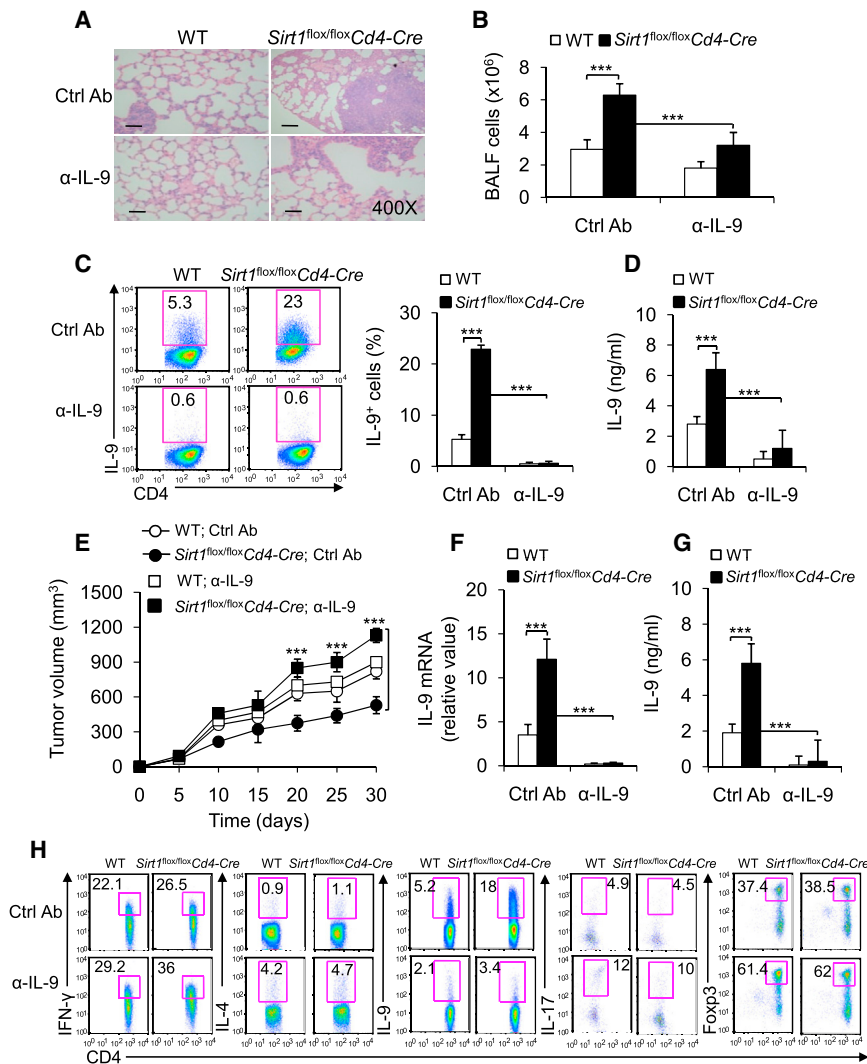


Figure 4. IL-9 Produced by SIRT1-Deficient CD4⁺ T Cells Is Involved in Allergic Airway Inflammation and Anti-tumor Immunity

(A–D) WT and *Sirt1^{fllox/fllox}Cd4-Cre* mice were sensitized and challenged intranasally with OVA on day 0, then treated with anti-IL-9 (α -IL-9) or isotype-matched control antibody (Ctrl Ab) every 3 days from day 0.

(A) Hematoxylin and eosin staining of paraffin-embedded sections of lungs from mice challenged as described above 48 hr after the final OVA challenge. Original magnification, $\times 400$.

(B) The absolute total cell number of BALF. (C) Intracellular IL-9 expression of CD4⁺ T cells isolated from lung DLNs of WT and *Sirt1^{fllox/fllox}Cd4-Cre* mice, with a typical figure shown (left) and the frequencies of positive cells are summarized (right).

(D) Secretion of IL-9 of CD4⁺ T cells isolated from the BALF of mice challenged as described above was measured with ELISA.

(E–H) *Rag1^{-/-}* mice given intravenous transfer of WT or *Sirt1^{fllox/fllox}Cd4-Cre* CD4⁺ T cells (stimulated via anti-CD3 and anti-CD28, together with TGF- β 1 and IL-4 for 3 days) along with simultaneous subcutaneous injection of B16 melanoma cells on day 0, then treated with anti-IL-9 (α -IL-9) or isotype-matched control antibody (Ctrl Ab) every 3 days from day 0.

(E) The tumor growth curve was plotted.

(F and G) Expression (F) or secretion (G) of IL-9 by CD4⁺ T cells sorted from the indicated mouse tumor-infiltrating cells and stimulated for 24 hr with anti-CD3. Expressions are presented relative to those that control gene *Gapdh*.

(H) Intracellular IFN- γ , IL-4, IL-17, and Foxp3 expression of CD4⁺ T cells isolated from tumor-infiltrating cells in WT and *Sirt1^{fllox/fllox}Cd4-Cre* mice, with a typical figure shown.

Data are representative of three independent experiments ($n = 3–10$). *** $p < 0.001$, compared with the indicated groups. See also Figures S3–S5.

acts via blocking hexokinase, the first rate-limiting enzyme of glycolysis, and the presence or absence of rapamycin, an mTOR inhibitor, which can block the mTOR-dependent metabolic pathway, including glycolysis, as a control. As expected, optimized doses of 2-DG or rapamycin significantly diminished the glycolytic activity (Figure 5A). Importantly, 2-DG and/or rapamycin treatment resulted in diminished ratio of IL-9⁺ in CD4⁺ T cells and IL-9 expression and secretion and restored the changes in the *Sirt1^{fllox/fllox}Cd4-Cre* cells (Figures 5B–5E). Taken together, these data indicate that glycolytic activity is required for SIRT1-directed Th9 cell differentiation.

SIRT1 Deficiency Promotes Th9 Cell Differentiation through an mTOR-HIF1 α -Dependent Glycolytic Pathway

How does SIRT1 regulate glycolytic activity in the course of directing Th9 cell differentiation? To elucidate the mechanism underlying SIRT1 function, we assessed the activation of the IL-4 and/or TGF- β 1 “downstream” pathways, including PU.1 (*Sfpi1*) (Chang et al., 2010), Smad3, STAT6, STAT5, mTOR, and HIF1 α . As expected, the Th9 cell-inducing conditions activated all of these pathways in WT T cells. In contrast, *Sirt1^{fllox/fllox}*

Cd4-Cre T cells exhibited activation of the PU.1, Smad3, STAT6, and STAT5 pathways as the WT cells did, but exhibited both a more potent and sustained activation of phosphorylation of the S6 ribosomal protein and transcription factor HIF1 α (Figures 6A and S6A–S6E). Consistent with this, modest overexpression of SIRT1 in T cells resulted in lower and less sustained inactivation of the phosphorylation of S6 ribosomal protein and HIF1 α (Figure 6B). Thus, SIRT1 is associated with the mTOR-HIF1 α metabolism axis.

To determine whether mTOR or HIF1 α is involved in this regulatory activity, we crossed the *Sirt1^{fllox/fllox}Cd4-Cre* and *Mtor^{fllox/fllox}* or *Hif1a^{fllox/fllox}* mice to conditionally delete *Sirt1* and *Mtor* or *Sirt1* and *Hif1a* gene expression in T cells, respectively (called *Sirt1^{-/-}Mtor^{-/-}* or *Sirt1^{-/-}Hif1a^{-/-}* mice hereafter). The increased IL-9⁺ cells and IL-9 mRNA expression or secretion in *Sirt1^{fllox/fllox}Cd4-Cre* cells are significantly restored in *Sirt1^{-/-}Mtor^{-/-}* cells or *Sirt1^{-/-}Hif1a^{-/-}* cells (Figures 6C–6E and 6F–6H). Consistent with this result, the increased glycolytic activity in *Sirt1^{fllox/fllox}Cd4-Cre* cells was also recovered in *Sirt1^{-/-}Mtor^{-/-}* cells or *Sirt1^{-/-}Hif1a^{-/-}* cells (Figure S6F). To further differentiate the role of mTORC1 and mTORC2, we applied

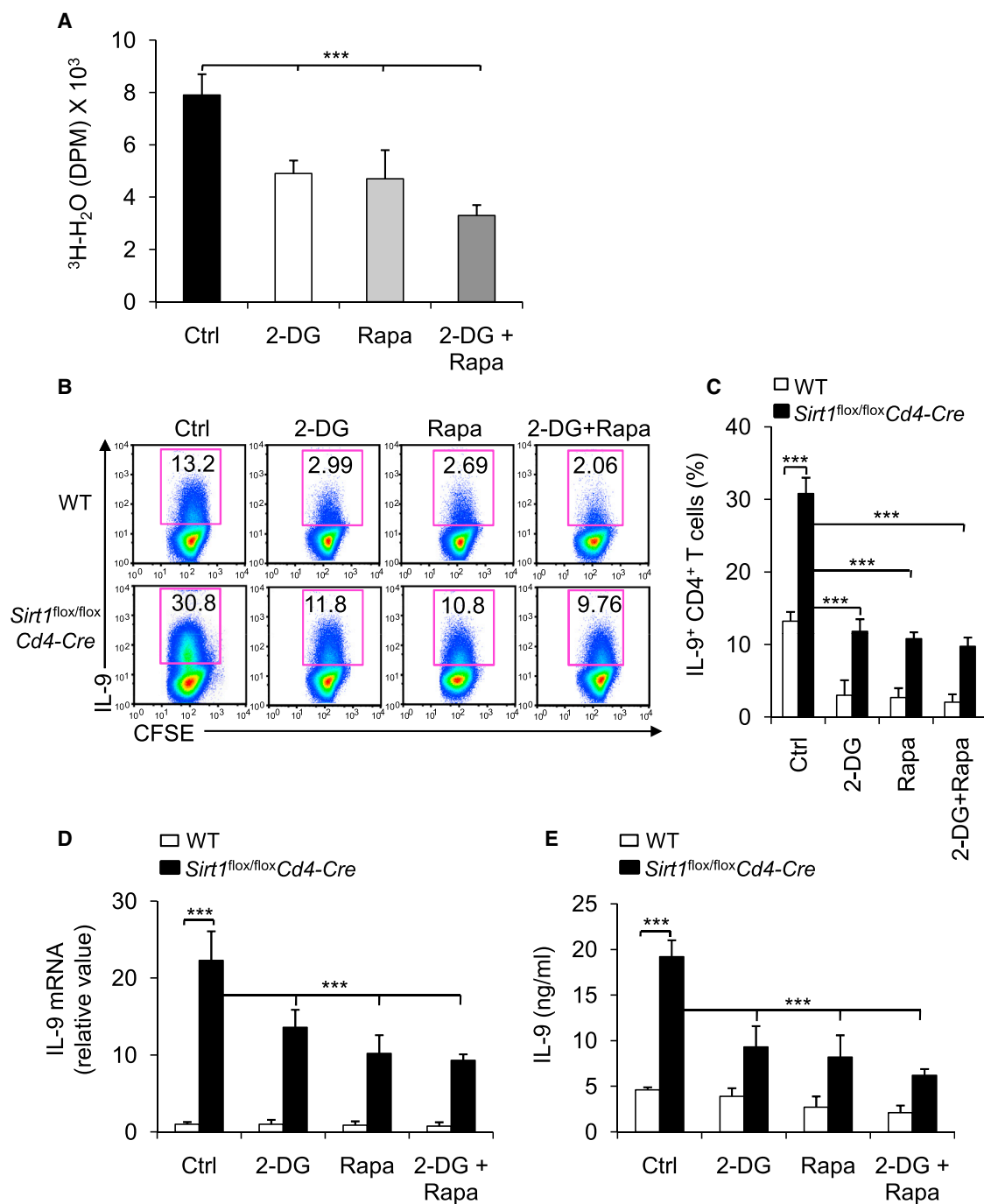


Figure 5. Blocking the Glycolytic Metabolism Pathway Restored SIRT1-Deficiency-Induced Th9 Cell Differentiation

(A) Naive T cells isolated from mouse spleen or peripheral lymph node (PLN) were differentiated under a Th9 cell-inducing conditions for 3 days in the presence of vehicle (Ctrl), 1 mM 2-DG, 50 nM rapamycin (Rapa), or 2-DG+Rapa. Glycolytic activity was measured.

(B and C) Naive T cells were labeled with CFSE and differentiated under Th9 cell-inducing conditions in the presence of vehicle, 2-DG, Rapa, or 2-DG+Rapa, followed by intracellular staining of IL-9. A typical figure is shown (B) and the frequencies of IL-9⁺ cells are summarized (C).

(D) IL-9 expression by the CD4⁺ T cells isolated from naive T cells in WT or *Sirt1*^{flx/flx} *Cd4-Cre* mice differentiated under Th9 cell-inducing conditions and stimulated for 24 hr with anti-CD3 for real-time PCR analyses. Expressions in the WT Ctrl groups were set to 1.

(E) The supernatant was collected and the IL-9 secretion was detected with ELISA

Data are representative of three independent experiments (n = 3–10). ***p < 0.001, compared with the indicated groups.

rapamycin with a dose (50 nM) that blocked the IL-9 production induced by SIRT1 deficiency and inhibited the phosphorylation of S6, indicative of mTORC1 activation. However, the phosphorylation of AKT Ser473, indicative of mTORC2 activation, was largely comparable between control and rapamycin-treated groups (Figures S6G–S6J). These results suggest that the mTORC1 signaling is largely responsible for mediating the IL-9 production induced by SIRT1 deficiency. Furthermore, *Sirt1^{flox/flox}Cd4-Cre*-induced HIF1 α was almost completely blocked in *Sirt1^{-/-}Mtor^{-/-}* cells, but blocking glycolysis with 2-DG did not affect the HIF1 α expression (Figures S7A and S7B). *Sirt1^{flox/flox}Cd4-Cre* and *Sirt1^{-/-}Mtor^{-/-}* or *Sirt1^{-/-}Hif1a^{-/-}* did not affect the proliferation and survival of Th9 cells (Figures S7C–S7E). Therefore, mTOR-HIF1 α -dependent glycolytic modulation is required for SIRT1-dependent Th9 cell differentiation.

HIF1 α Directly Binds to *Il9* in SIRT1-Deficiency-Induced Th9 Cells

We hypothesized that the transcription factor HIF1 α might exert an effect on the regulation of the *Il9* promoter in Th9 cells. To investigate this, we searched the *Il9* promoter for potential binding sites for HIF1 α using the Biobase database. We identified four overlapping putative binding sites for HIF1 α upstream of the transcription start site (TSS) (Figure 6I). To determine the *Il9* promoter occupancies by HIF1 α , binding motifs were used to design ChIP experiments. Primer sets flanking the HIF1 α binding at four sites in the *Il9* promoter were designed to amplify the immunoprecipitated ChIP DNA by real-time PCR (Figure 6I). Naive T cells isolated from WT and *Sirt1^{flox/flox}Cd4-Cre* mice were differentiated under Th9-cell-inducing conditions for 3 days and then analyzed by ChIP PCR. We found that HIF1 α bound to sites II and III in the *Il9* promoter in Th9 cells (Figure 6J). The pattern of HIF1 α binding was very similar to that of histone H3 (trimethylK4) antibodies, which supports the notion that these regions are in transcriptional regulation (Figure 6K).

To analyze the functional relevance of the binding of HIF1 α to its target sequences in the *Il9* locus, we investigated the ability of HIF1 α to regulate the activity of the *Il9* promoter in reporter assays. We used the reporter construct pGL4-*Il9*, which contains luciferase gene under the control of the *Il9* promoter. We found that cotransfection of the pGL4-*Il9* luciferase reporter construct with a plasmid encoding constitutively active HIF1 α (CA-HIF1 α) in 293T cells resulted in a dose-dependent significant increase in *Il9* transcription (Figure 6L). Therefore, these data show that binding HIF1 α to the *Il9* promoter activated *Il9* transcription, which was regulated by SIRT1.

SIRT1 Controls IL-9 Production in Human CD4⁺ T Cells

Next, we sought to apply a pharmacological approach to targets in human T cells for the genetic targeting of SIRT1. We first applied Ex527, a selective inhibitor of SIRT1, and SIRT1720, an activator of SIRT1 in human CD4⁺ T cells during Th9 cell induction. Consistent with the results from genetic targeting of SIRT1, we observed that a SIRT1 inhibitor and activator enhanced and reduced, respectively, the IL-9 mRNA expression and secretion, accompanied by alterations in the glycolytic molecule Glut1 (Figures 7A–7C). The experiment was then extended to include rapamycin (an inhibitor of mTOR), 2-ME (an inhibitor of HIF1 α),

and 2-DG (an inhibitor of the glycolytic pathway) to human T cells under Th9 cell-inducing conditions. Pharmacological inhibition of SIRT1 in human T cells largely recapitulated the observations in the genetic and pharmacological targeting of mouse CD4⁺ T cells in terms of the alterations of human T cell IL-9 expression, secretion, and glycolytic-associated molecules (Figures 7D–7F).

Consistent with the results in mouse cells, SIRT1 siRNA efficiently silenced SIRT1 expression in human CD4⁺ T cells and upregulated the expression of IL-9 and Glut1 (Figures 7G–7I). As expected, rapamycin, 2-ME, and 2-DG treatment largely reversed these effects (Figure 7H). Thus, the data show that SIRT1 mediates an evolutionarily conserved signaling pathway in both mice and humans.

DISCUSSION

T cell subsets differentiate from CD4⁺ T cells that have been exposed to a specialized cytokine environment. Compared with other T cell subsets that have been reported, Th9 cells are less well characterized. Recently, studies have reported factors that control their development, differentiation, and function (Palmer et al., 2015). Here we have shown that SIRT1-dependent signaling coupled to glycolytic activity negatively controlled the production of IL-9 in CD4⁺ T cells in allergic inflammation and anti-tumor immunity. First, TAK1 selectively suppressed SIRT1 during Th9 cell differentiation. Second, overexpression of SIRT1 reduced the amount of IL-9 in CD4⁺ T cells accompanied by lower glycolytic activity. Third, SIRT1 deficiency increased the amount of IL-9 in CD4⁺ T cells along with inducing a higher glycolytic activity. Fourth, SIRT1-mTOR-HIF1 α -dependent glycolytic activity was required for Th9 cell differentiation. Fifth, HIF1 α directly targeted the IL-9 gene promoter and controlled IL-9 production in CD4⁺ T cells.

Th9 cells are defined by their secretion of IL-9 (Kaplan et al., 2015). The production of IL-9 was first described in activated mouse T cells and subsequently cytokines that promote the differentiation of IL-9-producing T cells in culture were identified (Schmitt et al., 1994). Th9 cells have been shown to have roles in various diseases, including allergic inflammation (Cheng et al., 2002) and tumors (Purwar et al., 2012). Mouse studies have provided strong evidence that Th9 cells exert anti-tumor effects, particularly in melanoma (Kim et al., 2015). Here we have demonstrated that SIRT1 deficiency promotes IL-9 production in CD4⁺ T cells and contributes to the responses of allergic airway inflammation and anti-tumor immunity.

SIRT1 is a nuclear NAD⁺-dependent histone deacetylase as well as a key transcriptional regulator of inflammation (Yang et al., 2015). In innate immune cells, SIRT1 has been identified as an important repressor of inflammation (Liu et al., 2015). In T cells, SIRT1 is required to maintain T cell tolerance (Yang et al., 2015). Distinct metabolic programs support the differentiation of CD4⁺ T cells into their separate subsets. However, whether SIRT1 is involved in the metabolism-dependent modulation of intrinsic T cell subset differentiation was not clear. Our data show that TAK1 mediated the selective suppression of SIRT1 in Th9 cells and that the modulation of SIRT1-dependent signaling coupled with glycolytic metabolism is responsible for Th9 cell differentiation and contributed to

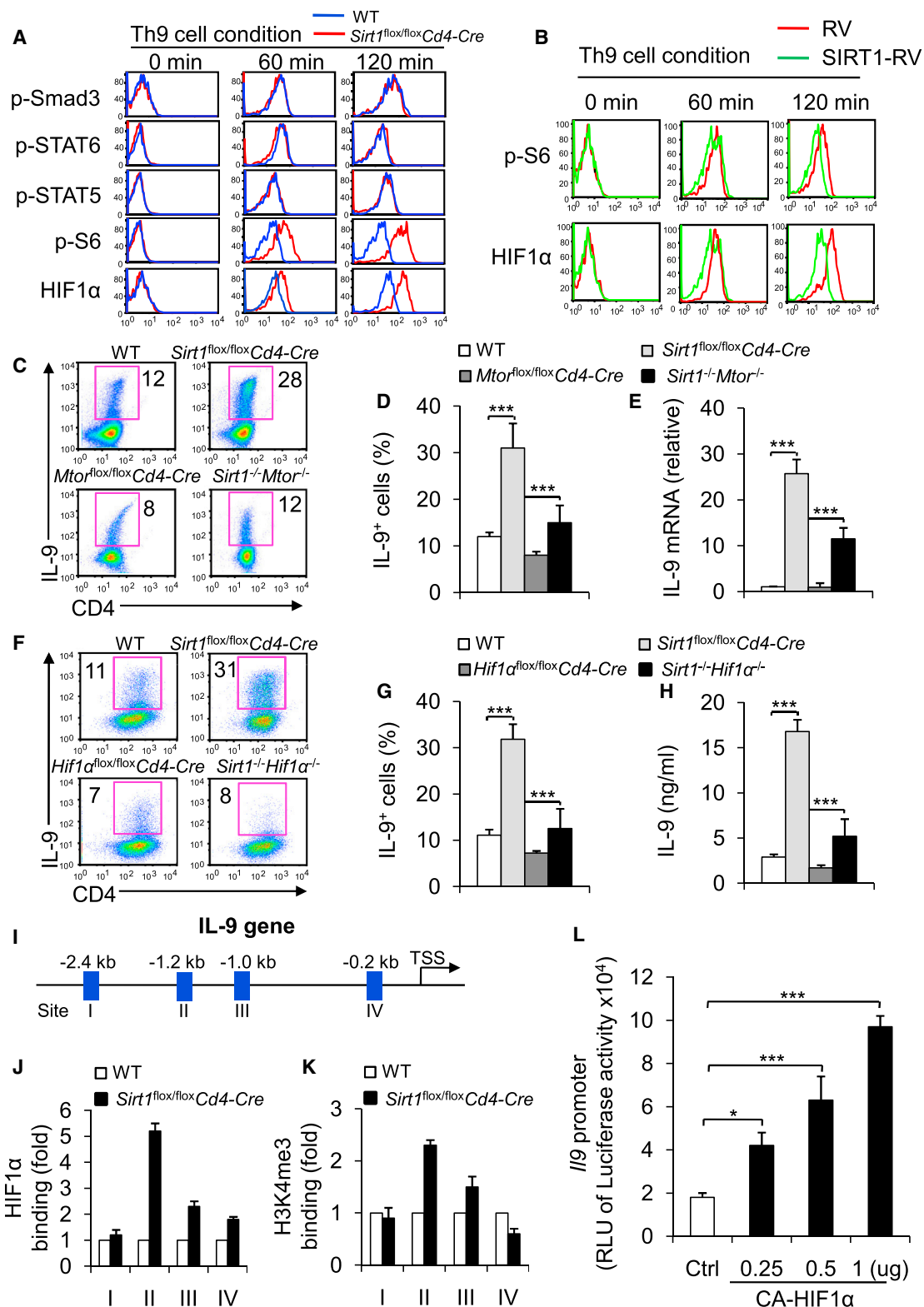


Figure 6. SIRT1 Deficiency Promotes Th9 Cell Differentiation through mTOR-HIF1 α -Dependent Signaling Coupled to the Glycolytic Pathway
(A) Activation of the downstream signaling pathways of IL-4 or TGF- β or metabolism. Intracellular staining of the phosphorylation of Smad3, STAT6, STAT5, and S6 and the expression of HIF1 α in the CD4⁺ T cells isolated from WT and *Sirt1^{fllox/flox}Cd4-Cre* mice under Th9 cell-inducing conditions.
(B) Intracellular staining of the phosphorylation of S6 and the expression of HIF1 α by eGFP⁺ cells sorted from Th9 cell-inducing conditions transduced with RV or SIRT1-RV.

(legend continued on next page)

Th9 cell-associated allergic airway inflammation and anti-tumor immune response.

HIF1 α is a key transcription factor that orchestrates the expression of glycolytic enzymes that can be induced under normoxia as well as hypoxia and plays a central role in pro-inflammatory cytokine production (Cramer et al., 2003). Th17 cells have been shown to rely on glycolysis for their development and maintenance by mTOR-HIF1 α pathway (Shi et al., 2011). HIF1 α deficiency increases the generation of Treg cells, and blocking glycolysis inhibits Th17 cell differentiation (Dang et al., 2011; Shi et al., 2011). Recent studies have shown that SIRT1 directly inactivates HIF1 α through the direct interplay between SIRT1 and HIF1 α in mouse tissues, even in the absence of hypoxia (Lim et al., 2010). SIRT1-HIF1 α axis confers a beneficial effect against certain diseases (Cheng et al., 2014). Consistent with a previous report, although Th9 cell-inducing condition is not optimized for driving Th17 and Treg cell differentiation, HIF1 α upregulation induced by SIRT1 deficiency reciprocally reduced the percentage of Treg cells and increased the percentage of Th17 cells. Importantly, in this condition, SIRT1 deficiency preferentially promoted IL-9 production in CD4⁺ T cells through HIF1 α -dependent glycolysis pathway. Moreover, HIF1 α , downstream of SIRT1-mTOR, directly binds to the *Il9* promoter to promote *Il9* transcription. However, blocking glycolysis did not alter the HIF1 α expression. This suggests that, in addition to HIF1 α 's role in regulating glycolysis, which provides crucial bioenergetics support to drive Th9 cell differentiation, HIF1 α also directly bound to the IL-9 gene promoter to drive IL-9 transcription. Thus, these data suggest that heightened HIF1 α signaling promotes glycolysis and also directly enhances IL-9 expression, both of which are required for SIRT1-directed Th9 cell differentiation.

mTOR is responsible for the glycolytic response upstream of HIF1 α (Cheng et al., 2014). SIRT1 negatively controls mTOR (Liu et al., 2014). mTOR suppression by rapamycin or mTOR deletion promoted the generation of Treg cells (Delgoffe et al., 2009). Th1, Th2, or Th17 cells strongly engage glycolysis via mTOR, whereas Treg cells depend more on the oxidation of lipids (Zeng and Chi, 2015). Consistent with previous studies, we found that SIRT1 deficiency in Th9 cells, but not other T cell subsets, upregulates p-S6 expression, a downstream target of mTOR. Moreover, *Sirt1*^{-/-}*Mtor*^{-/-} cells almost

completely rescue the Th9 cell differentiation and glycolysis activities in *Sirt1*^{fllox/fllox}*Cd4-Cre* cells and also largely block HIF1 α expression. Thus, TAK1-SIRT1-mTOR-HIF1 α signaling coupled with glycolytic pathway is critical for the differentiation of Th9 cells.

Altogether, our work suggested that SIRT1 is selectively suppressed by TAK1 during Th9 cell differentiation and might function as a gatekeeper of downstream mTOR-HIF1 α axis. As such, mTOR-HIF1 α -IL-9 promoter transcriptional regulation coupled with glycolytic activity mechanism modulation is selective for SIRT1-dependent differentiation of Th9 cells that confer protection against tumors and are involved in allergic airway inflammation.

EXPERIMENTAL PROCEDURES

Mice

All animal experiments were performed with the approval of the Animal Ethics Committee of Beijing Normal University, Beijing, China and Fudan University, Shanghai, China. *Sirt1*^{fllox/fllox}, *Mtor*^{fllox/fllox}, *Hif1a*^{fllox/fllox}, and *Cd4-Cre* mice have been described previously (Liu et al., 2009, 2015) and were extensively backcrossed to the C57BL/6 background. WT controls for *Sirt1*^{fllox/fllox}*Cd4-Cre* mice (*Cd4-Cre*; *Sirt1*^{fllox/fllox}) included *Cre*⁺ mice (*Cd4-Cre*; *Sirt1*^{+/+}) to account for the effects of *Cre*. *Rag1*^{-/-} mice (on a C57BL/6 background) were from the Jackson Laboratory. Sex-matched littermates at 7–10 weeks of age were used in the experiments described in this study. All mice were bred and maintained under specific-pathogen-free conditions.

Induction of Allergic Airway Inflammation

Allergic airway inflammation was induced as previously described (Cheng et al., 2002). Mice were sensitized by intraperitoneal injection on days 0 and 14 of OVA (Sigma-Aldrich) adsorbed with aluminum hydroxide (Sigma-Aldrich) at a dose of 10 μ g OVA per 1 mg aluminum hydroxide. From day 21, mice were exposed to aerosolized OVA (1% w/v) for 30 min per day for 5–6 consecutive days. Mice were sacrificed 48 hr after the final intranasal challenge. To evaluate the effects of anti-IL-9 on mouse allergic airway inflammation, mice were given 20 μ g mouse IgG2a (R&D Systems) or anti-IL-9 (R&D Systems) intravenously 30 min before the challenges every day from day 20 to day 24.

Tumor-Bearing Model

Naive T cells were isolated from *Sirt1*^{fllox/fllox}*Cd4-Cre*⁺ and *Sirt1*^{+/+}*Cd4-Cre*⁺ mice, then cultured for 3 days with anti-CD3 and anti-CD28 (both identified above) together with IL-4 and TGF- β 1. These cells were collected and then transferred intravenously into *Rag1*^{-/-} mice (5 $\times$ 10⁶ cells per mouse) on day 0. On the same day, B16 melanoma cells (5 $\times$ 10⁵ cells per mouse) were injected subcutaneously into the side of the abdomen of the mice given

(C and D) Naive T cells were differentiated under Th9 cell-inducing conditions in WT, *Sirt1*^{fllox/fllox}*Cd4-Cre*, *Mtor*^{fllox/fllox}*Cd4-Cre*, or *Sirt1*^{-/-}*Mtor*^{-/-} mice, followed by intracellular staining of IL-9. A typical figure is shown in (C) and the frequencies of IL-9⁺ cells summarized in (D).

(E) IL-9 expression by CD4⁺ T cells isolated from naive T cells in the indicated mice differentiated under Th9 cell-inducing conditions and stimulated for 24 hr with anti-CD3 for real-time PCR analyses. Expressions in WT were set to 1.

(F) Naive T cells were differentiated under Th9 cell-inducing conditions in WT, *Sirt1*^{fllox/fllox}*Cd4-Cre*, *Hif1a*^{fllox/fllox}*Cd4-Cre*, or *Sirt1*^{-/-}*Hif1a*^{-/-} mice, followed by intracellular staining of IL-9. A typical figure is shown in (F) and the frequencies of IL-9⁺ cells are summarized in (G).

(H) IL-9 secretion by CD4⁺ T cells isolated from naive T cells obtained from the indicated mice differentiated under Th9 cell-inducing conditions and stimulated for 24 hr with anti-CD3 for ELISA.

(I) Structural schematics of the four predicated binding sites for HIF1 α in the *Il9* promoter.

(J) ChIP analysis of HIF1 α binding to the *Il9* promoter. Regulation of HIF1 α binding to the four sites within the *Il9* promoter. CD4⁺ T cells from WT and *Sirt1*^{fllox/fllox}*Cd4-Cre* mice were differentiated under Th9 cell-inducing conditions and ChIP-SYBR Green PCR was performed to determine HIF1 α binding to the *Il9* promoter. The Abs are anti-HIF1 α and control IgG. The total input DNA was used for normalization of the data.

(K) ChIP analysis of H3K4 modifications normalized to input controls at the HIF1 α binding four sites within the *Il9* promoter under Th9 cell-inducing conditions for 2 days.

(L) HEK293T cells were transfected with active HIF1 α (CA-HIF1 α) together with a constant amount of an *Il9* promoter-luciferase vector. Cells were lysed 24 hr later, and then the luminescence was measured.

Data are representative of three independent experiments (n = 3–10). *p < 0.05 and ***p < 0.001, compared with the indicated groups. Please see Figures S6 and S7.

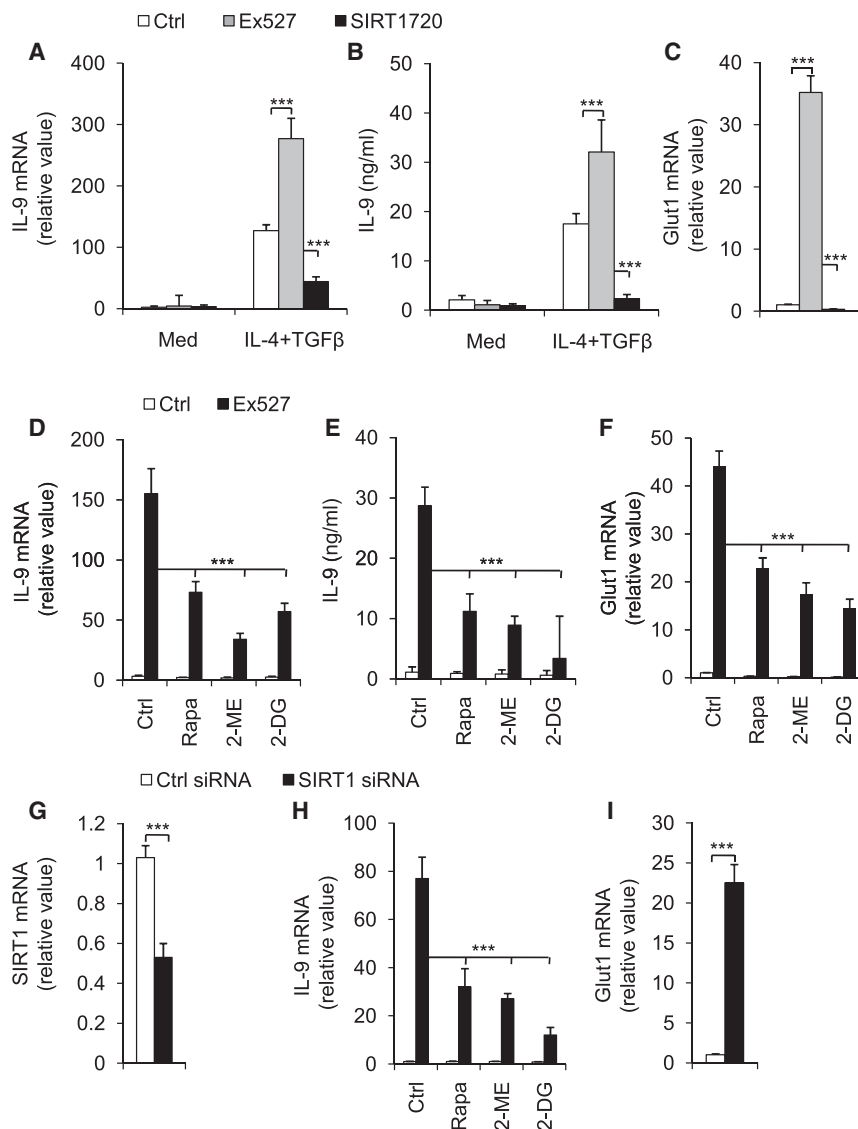


Figure 7. SIRT1 Controls IL-9 Production in Human CD4⁺ T Cells

Naive CD4⁺CD25[−]CD45RA⁺ cells isolated from human peripheral blood mononuclear cells and stimulated for 48 hr with anti-CD3, anti-CD28, and vehicle (medium; med) or with IL-4 and TGF-β1 and the indicated treatment.

(A and D) IL-9 mRNA expression was measured by real-time PCR.

(B and E) IL-9 secretion of supernatant as in (A).

(C and F) Glut1 mRNA expression was analyzed.

(G–I) Expressions of SIRT1 mRNA (G), IL-9 mRNA (H), and Glut1 mRNA (I) in human naive CD4⁺ T cells transfected with control siRNA (Ctrl siRNA) or SIRT1-specific siRNA (SIRT1 siRNA) and stimulated for 48 hr with anti-CD3 and anti-CD28 plus TGF-β1 and IL-4 and the indicated treatment (expressions in Ctrl siRNA were set to 1).

Retroviral Transduction and RNA Interference of SIRT1

SIRT1 was cloned into the MSCV retroviral vector (Clontech Laboratories), as previously described (Liu et al., 2010). A gene-silencing lentiviral construct was generated by subcloning the gene-specific siRNA sequence into a lentiviral siRNA expression plasmid (pMagic4.1; Sbo-bio). The SIRT1 siRNA sequence of 5'-CAGGTGTCAGGA ATCCAAAG-3' was synthesized (Takala) (Liu et al., 2014).

RNA and Protein Analyses

Real-time PCR was performed with probe sets from Applied Biosystems and normalized against the endogenous control gene (Liu et al., 2015). Immunoblot was performed with the following antibodies: p-S6 and SIRT1 (both from Cell Signaling Technology), HIF1α (R&D Systems), and β-actin (Sigma), as described previously (Liu et al., 2014).

ChIP and Luciferase Assay

Chromatin immunoprecipitation (ChIP) was performed according to the protocol described previously (Lim et al., 2010). Luciferase reporter assays were also performed as described previously

(Dang et al., 2011). The mouse *I/9* promoter region (−2410 to +32) was amplified from genomic DNA by PCR with the forward primer (5'-TCGACGCGTGAT GACCTGTgacctgAC-3') and the reverse primer (5'-TCCcggggcaGAGGC AAGGATGTATGT-3'). The lowercase letters in the primers were Hind III and KpnI sites that were introduced to allow forced cloning into the pGL4 basic luciferase reporter gene vector (Promega).

Glycolysis Flux Assay

Glycolysis in T cells was determined by measuring the detritiation of [3-³H]-glucose (PerkinElmer) (Liu et al., 2014).

Statistical Analyses

All data are presented as the mean ± SD. Student's unpaired t test for comparison of means was used to compare differences between groups. A p value (alpha-value) of less than 0.05 was considered to be statistically significant.

ACCESSION NUMBERS

The microarray data were deposited into the GEO database under accession number GSE79607.

the transfer of T cells. Mice were treated intraperitoneally with anti-IL-9 or control mouse IgG2a (both identical to the above) every 3 days from day 0, as described previously (Nakatsukasa et al., 2015).

Cell Purification and Culture

Lymphocytes were isolated from the spleen and lymph nodes of mice and sorted on a FACSaria II (Becton Dickinson). The sorted naive T cells (CD4⁺TCR⁺CD62L^{hi}CD44^{lo}CD25[−]) were used for in vitro culture as described (Liu et al., 2010). T cells were activated with 2 μg/mL anti-CD3 (2C11; Bio X Cell), 2 μg/mL anti-CD28 (37.51; Bio X Cell), and 100 U/mL human IL-2 (R&D Systems). For Th9 cell differentiation, cultures were supplemented with 10 ng/mL IL-4 (R&D Systems), 2 ng/mL TGF-β1 (R&D Systems), and, for certain experiments only, TGF-β1 and IL-4, as indicated. After 5–6 days culture, differentiated T cells were restimulated for 24–48 hr with plate-bound anti-CD3 (1 μg/mL), and the cell-free supernatants collected after centrifugation were stored at −80°C until use. The amount of IL-9 produced was determined by ELISA (BD Bioscience). T cell proliferation was determined by pulsing the cells with [³H] thymidine for the final 12–16 hr of culture or by labeling with CFSE (carboxyfluorescein diacetate succinimidyl diester; Invitrogen). Flow cytometry was performed with antibodies from eBioscience or BD Biosciences.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.immuni.2016.05.009>.

AUTHOR CONTRIBUTIONS

Y.W. and Y.B. designed and conducted the experiment with cells and mice and analyzed data; X.C. and C.L. did some gene expression analysis; Y. Li, Z.Z., J.W., Y. Lu, Q.Y., H.S., and H.Y. assisted with analysis and interpretation of experiments and results; and G.L. developed the concept, designed and conducted the experiments, analyzed data, wrote the manuscript, and provided overall direction.

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Supplemental Information

Histone Deacetylase SIRT1

Negatively Regulates the Differentiation

of Interleukin-9-Producing CD4⁺ T Cells

Yu Wang, Yujing Bi, Xi Chen, Chunxiao Li, Yan Li, Zhengguo Zhang, Jian Wang, Yun Lu, Qing Yu, Huilin Su, Hui Yang, and Guangwei Liu

Supp. Fig. 1

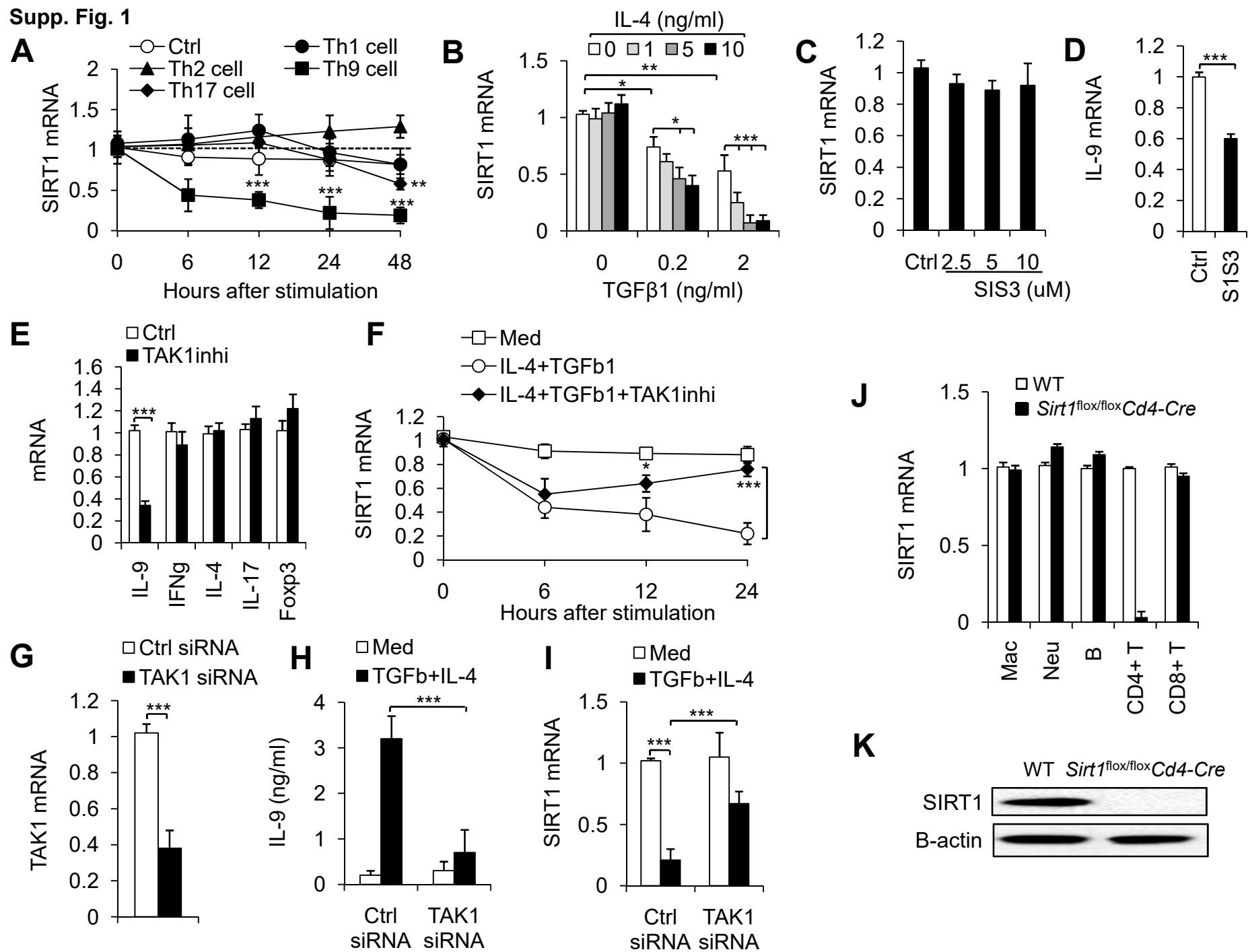


Figure S1. SIRT1 expression in WT or *Sirt1*^{flox/flox}*Cd4-Cre* mice. Related to Fig.1.

(A) SIRT1 mRNA expression as examined by qPCR analyses in T cells activated by various T cell subset-inducing conditions for different time course (Expressions in naïve T cells were set to 1). (B) Naïve T cells were activated by anti-CD3/CD28 in the presence of various concentration of TGFβ1 and/or IL-4 for 24 h and SIRT1 expression was analyzed by qPCR (Expressions in activated without TGFβ1 and IL-4 were set to 1). (C-D) Naïve T cells were differentiated for 3 d under Th9 cell condition in the presence of S1S3 (an inhibitor of Smad3) and SIRT1 (C) and IL-9 mRNA (D) were analyzed (Expressions in Ctrl groups were set to 1). (E) Indicated factors were determined under various T cell subset conditions for 3 d in the presence or absence of TAK1 inhibitor (5z-7-oxozeaenol, sigma, 50 nM). Expressions in Ctrl groups were set to 1. (F) Naïve T cells were activated by indicated condition for different time course and SIRT1 mRNA expression was determined by qPCR. Expressions in naïve T cells were set to 1. (G-I) Expression of TAK1 (G), IL-9 production (H) and SIRT1 mRNA expression (I) in naïve CD4⁺ T cells transfected with control siRNA (Ctrl siRNA), or TAK1-specific siRNA (TAK1 siRNA), and stimulated for 3 d with anti-CD3 and anti-CD28 plus TGFβ1 and IL-4 (Presented in the same manner as in F. Expressions in Ctrl siRNA at med groups were set to 1). (J) Macrophages (CD11b⁺F4/80⁺), Neutrophils (CD11b⁺Ly6G⁺), B cells (CD19⁺), CD4⁺ T cells (CD4⁺ TCR⁺), and CD8⁺ T cells (CD8⁺ TCR⁺) were isolated from WT or *Sirt1*^{flox/flox}*Cd4-Cre* mouse spleen and the purity was confirmed by flow cytometry, and SIRT1 expressions were determined by qPCR (Expressions in WT were set to 1). (K) CD4⁺ T cells isolated from WT and *Sirt1*^{flox/flox}*Cd4-Cre* mice and SIRT1 expressions were determined by immunoblot. Data in Fig. S1 are shown as mean ± SD, n=3-4, from one of three independent experiments. **P*<0.05, ***P*<0.01 and ****P*<0.001, compared with the indicated groups.

Supp. Fig. 2

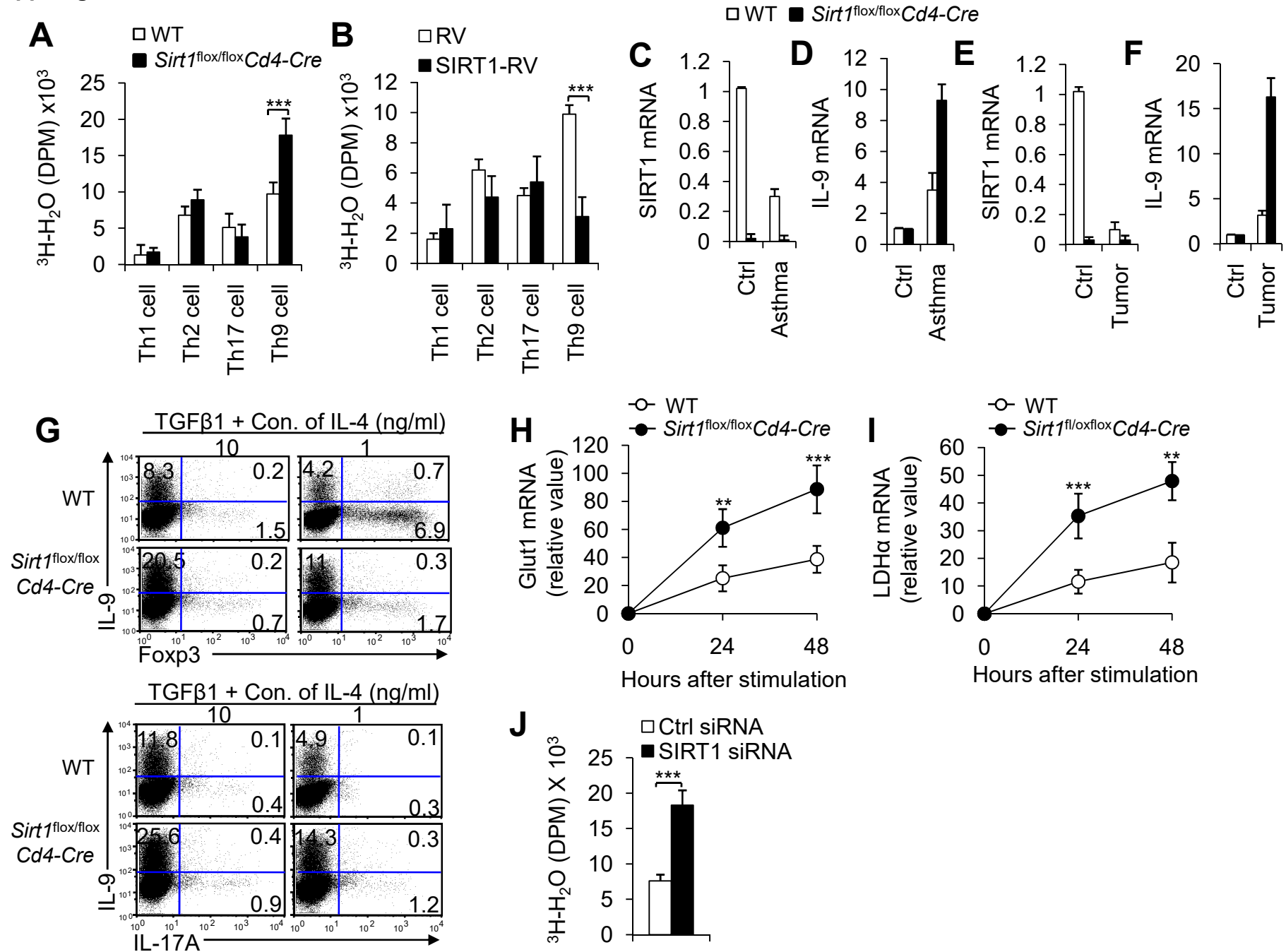


Figure S2. SIRT1 deficient or silencing promotes IL-9 expressions and enhances the glycolytic activity of T cells under Th9 cells-inducing condition. Related to Fig. 1 and 3.

(A) Naïve T cells from WT and *Sirt1*^{flox/flox}*Cd4-Cre* mice were differentiated under various T cell subset-inducing conditions, followed by measured of glycolytic activity. (B) eGFP⁺ cells sorted from various T cell subset-inducing condition transduced with control retrovirus (RV) or *Sirt1*-expressing retrovirus (SIRT1-RV) were measured for glycolytic activity. (C-D) Mice were sensitized intraperitoneal injection on days 0 and 14 of ovalbumin (OVA, Sigma-Aldrich) adsorbed with aluminum hydroxide (Sigma-Aldrich) at a dose of 10 µg OVA per 1 mg aluminum hydroxide. From day 21, mice were exposed to aerosolized OVA (1% w/v) for 30 min per day for 5-6 consecutive days. Mice were killed 48 h after the final intranasal challenge, T cells were isolated from lung draining lymph node (DLN) and SIRT1 mRNA (C) and *Il9* mRNA (D) were measured with qPCR (Expressions of Ctrl WT groups were set to 1). (E-F) *Rag1*^{-/-} mice given intravenous transfer of WT or *Sirt1*^{flox/flox}*Cd4-Cre* naïve CD4⁺ T cells (stimulated via anti-CD3 and anti-CD28, together TGFβ1 and with IL-4 for 3 d) along with simultaneous subcutaneous injection of B16 melanoma cells on day 0. After 30 days, CD4⁺ T cells were isolated from mouse tumor tissue in WT or *Sirt1*^{flox/flox}*Cd4-Cre* mice and SIRT1 mRNA (E) and *Il9* mRNA (F) were measured with qPCR (Expressions of Ctrl WT groups were set to 1). (G) Naïve T cells from WT and *Sirt1*^{flox/flox}*Cd4-Cre* mice were differentiated under Th9 cell-inducing conditions, followed by measured of the expressions of IL-9 and Foxp3 (upper panel) or IL-9 and IL-17 (lower panel) with intracellular staining method. (H-I) Naïve T cells from WT and *Sirt1*^{flox/flox}*Cd4-Cre* mice were differentiated under Th9 cell conditions, followed by measured of mRNA expression of glycolytic molecules and showed the time course changes of glycolytic molecules (Expressions in naïve T cells were set to 1). (J) Naïve CD4⁺ T cells from WT or *Sirt1*^{flox/flox}*Cd4-Cre* mice transfected with control siRNA (Ctrl siRNA) or SIRT1-specific siRNA (SIRT1 siRNA) and stimulated for 48 h with anti-CD3 and anti-CD28 plus TGFβ1 and IL-4, followed by measured of glycolytic activity. Data in Fig. S2 are shown as mean ± SD, n=3-5, from one of three independent experiments. ***P*<0.01 and ****P*<0.001, compared with the indicated groups.

Supp. Fig. 3

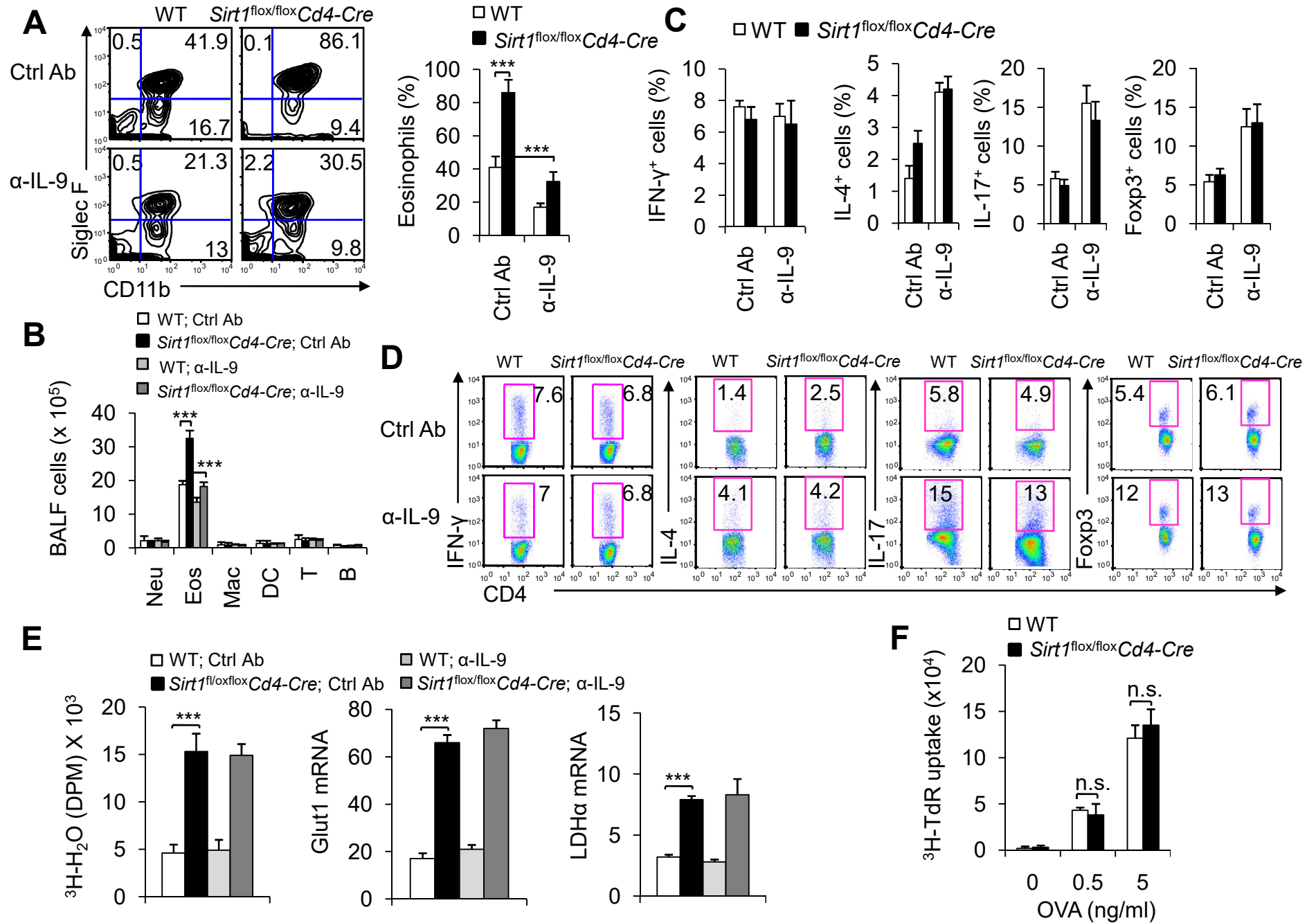


Figure S3. IL-9 produced by SIRT1 deficient CD4⁺ T cells is involved in the development of allergic airway inflammation. Related to Fig. 4.

Mice were sensitized intraperitoneal injection on days 0 and 14 of ovalbumin (OVA, Sigma-Aldrich) adsorbed with aluminum hydroxide (Sigma-Aldrich) at a dose of 10 µg OVA per 1 mg aluminum hydroxide. From day 21, mice were exposed to aerosolized OVA (1% w/v) for 30 min per day for 5-6 consecutive days. Mice were killed 48 h after the final intranasal challenge. **(A-E)** WT and *Sirt1^{flox/flox}Cd4-Cre* mice sensitized and challenged intranasally with OVA on day 0, then treated with anti-IL-9 (α-IL-9) or isotype-matched control antibody (Ctrl Ab) every 3 d from day 0. **(A)** The percentages of eosinophils in BALF fluids of WT and *Sirt1^{flox/flox}Cd4-Cre* were detected by flow cytometry (left), and the frequencies of eosinophils were summarized (right). **(B)** The indicated cell types were determined with flow cytometry and the absolute cell number were summarized. **(C-D)** Intracellular IFN-γ, IL-4, IL-17 and Foxp3 expression of CD4⁺ T cells isolated from lung DLN in WT and *Sirt1^{flox/flox}Cd4-Cre* mice and the frequencies of positive cells were summarized **(C)**, and typical figures were showed **(D)**. **(E)** CD4⁺ T cells isolated from lung DLN, followed by measured of glycolytic activity and mRNA expression of indicated glycolytic molecules. Expressions are presented relative to those of the control gene *Gapdh*. **(F)** WT and *Sirt1^{flox/flox}Cd4-Cre* mice were killed 48 h after the final intranasal challenge, proliferation of DLN cells after *ex vivo* stimulation with OVA for 72 h. Data in Fig. S3 are representative of three independent experiments (n=3-4).

****P*<0.001, compared with the indicated groups. n.s., no significant.

Supp. Fig. 4

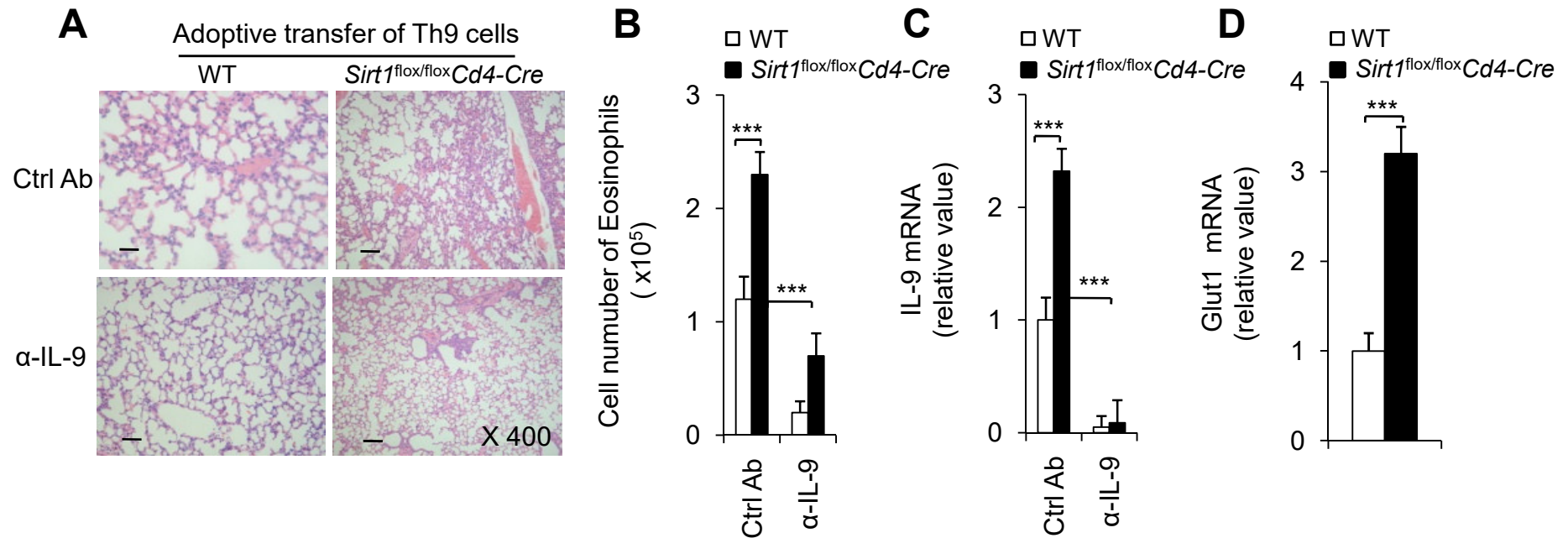


Figure S4. IL-9 produced by SIRT1 deficient CD4⁺ T cells is involved in the development of allergic airway inflammation. Related to Fig. 4.

Rag1^{-/-} mice (n=16) were adoptively transferred with Th9 cells and was sensitized and challenged intranasally with OVA on day 0, then treated with anti-IL-9 (α -IL-9) or isotype-matched control antibody (Ctrl Ab) every 3 d from day 0. **(A)** Hematoxylin and eosin staining of paraffin-embedded sections of lungs from mice challenged as described above 24 h after the final OVA challenge. Original magnification, x 400. **(B)** The absolute total cell number of Eosinophils in BALF. Infiltrating CD4⁺ T cells from lung DLN were isolated and stimulated with anti-CD3 for 24 h, and IL-9 mRNA **(C)** and Glut1 mRNA **(D)** expressions of CD4⁺ T cells were measured by qPCR. Expressions in WT (Ctrl Ab) were set to 1. Data in Fig. S4 are representative of two independent experiments (n=16).

****P*<0.001 compared with the indicated groups.

Supp. Fig. 5

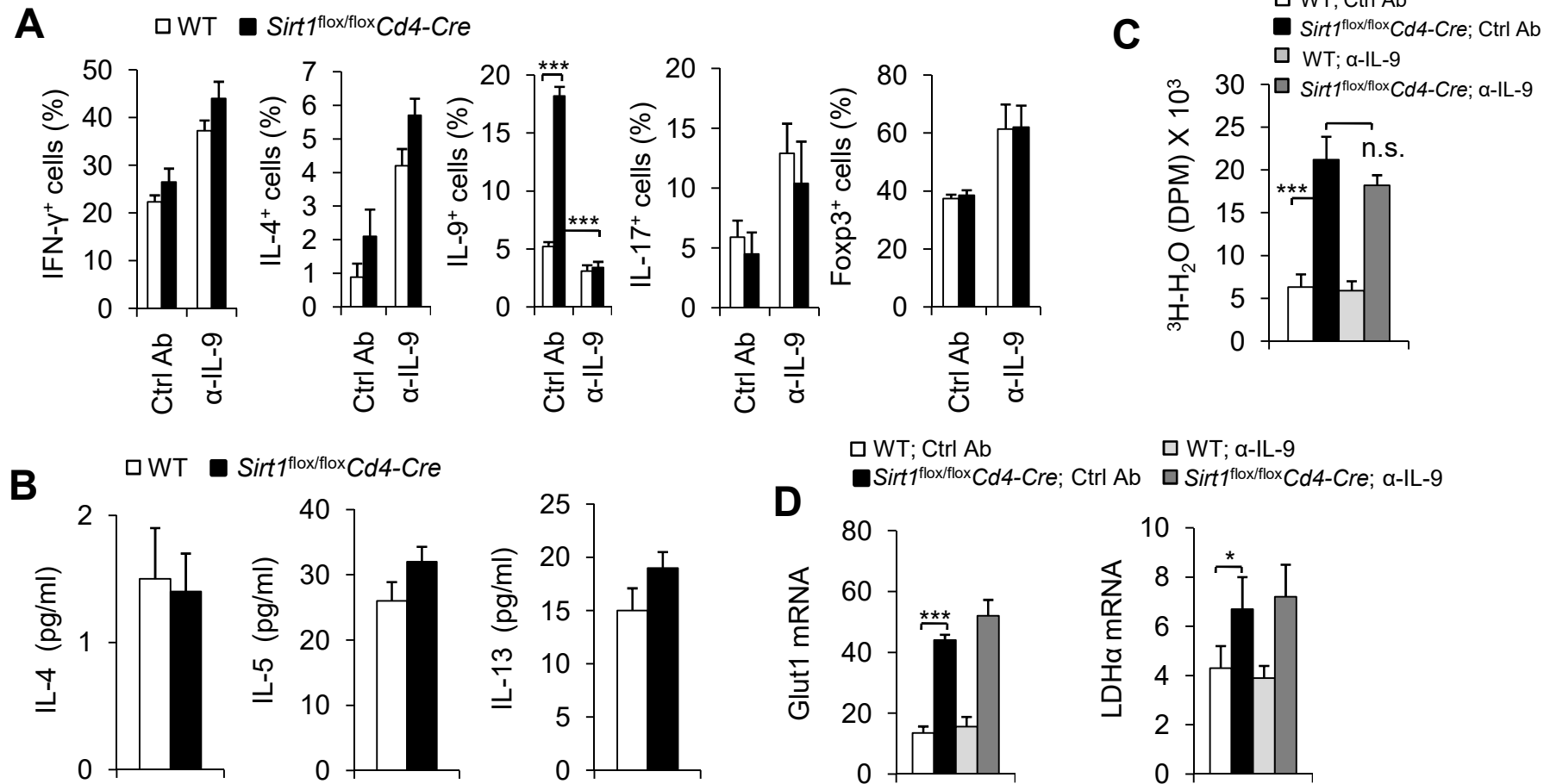


Figure S5. IL-9 produced by SIRT1 deficient CD4⁺ T cells is involved in delaying tumor growth. Related to Fig. 4.

Rag1^{-/-} mice given intravenous transfer of WT or *Sirt1*^{flox/flox} Cd4-Cre naïve CD4⁺ T cells (stimulated via anti-CD3 and anti-CD28, together TGF β 1 and with IL-4 for 3 d) along with simultaneous subcutaneous injection of B16 melanoma cells on day 0, then treated with anti-IL-9 (α -IL-9) or isotype-matched control antibody (Ctrl Ab) every 3 d from day 0. **(A)** Intracellular IFN- γ , IL-4, IL-9, IL-17, and Foxp3 expression of CD4⁺ T cells isolated from tumor infiltrating cells in WT and *Sirt1*^{flox/flox} Cd4-Cre mice and the frequencies of positive cells were summarized. **(B)** CD4⁺ T cells were isolated from solid tumor tissue and were stimulated for 72 h with anti-CD3 and anti-CD28 (identical with above). ELISA of indicated cytokine in supernatant of CD4⁺ T cells. **(C)** CD4⁺ T cells isolated from WT and *Sirt1*^{flox/flox} Cd4-Cre mouse tumor, followed by measured of glycolytic activity. **(D)** CD4⁺ T cells were isolated from solid tumor tissue and followed by measured of mRNA expression of indicated glycolytic molecules. Data in Fig. S5 are representative of three independent experiments (n=3-5). **P*<0.05, and ****P*<0.001, compared with the indicated groups. n.s., no significant.

Supp. Fig. 6

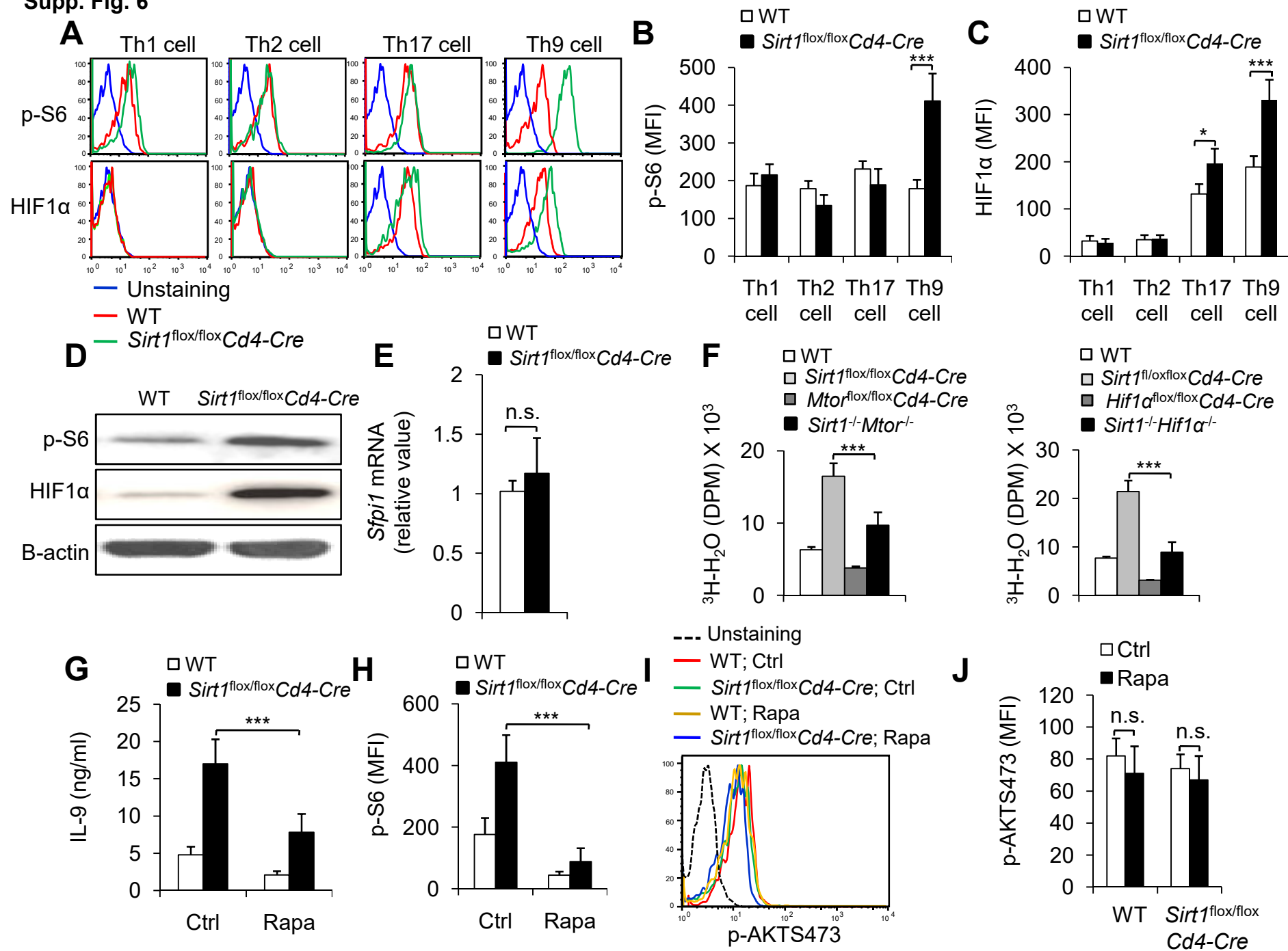


Figure S6. mTOR and HIF1 α -dependent glycolysis is upregulated by SIRT1 deficiency CD4⁺ T cells under Th9 cell-inducing condition. Related to Fig.6.

(A-C) p-S6 and HIF1 α expression of various T cell subset. Intracellular staining of phosphorylation of S6, and expression of HIF1 α in the CD4⁺ T cells isolated from WT and *Sirt1*^{flox/flox}*Cd4-Cre* mice under various T cell subset-inducing condition. Typical figure showed in A, and the MFI of p-S6 (B) and HIF1 α (C) of T cells summarized. (D) Immunoblot of phosphorylation of S6 and expression of HIF1 α in the CD4⁺ T cells isolated from WT and *Sirt1*^{flox/flox}*Cd4-Cre* mice under Th9 cell-inducing condition. (E) The mRNA expression of *Sfp1* (gene encoding PU.1) by WT or *Sirt1*^{flox/flox}*Cd4-Cre* mouse CD4⁺ T cells under Th9 cell-inducing condition and stimulated for 24 h with anti-CD3. Expressions in WT groups were set to 1. (F) Naïve T cells were differentiated under Th9 cell conditions in WT, *Sirt1*^{flox/flox}*Cd4-Cre*, *Mtor*^{flox/flox}*Cd4-Cre* or *Sirt1*^{-/-}*Mtor*^{-/-} mice (left panel) or in WT, *Sirt1*^{flox/flox}*Cd4-Cre*, *Hif1* α ^{flox/flox}*Cd4-Cre* or *Sirt1*^{-/-}*Hif1* α ^{-/-} mice (right panel) followed by measured of glycolytic activity. (G-J) Naïve CD4⁺ T cells isolated from WT or *Sirt1*^{flox/flox}*Cd4-Cre* mice were differentiated under Th9 cell-inducing conditions for 3 d in the presence of vehicle (Ctrl), 50 nM rapamycin (Rapa). The CD4⁺ T cells were stimulated for 24 h with anti-CD3, supernatant was collected, and IL-9 secretion was determined with ELISA (G). Intracellular staining of the phosphorylation of S6 (H) and AKT Ser473 (I-J) in the CD4⁺ T cells. The typical figure of p-AKT Ser473 showed in I. The MFI of p-S6 or p-AKT Ser473 of T cells summarized (H or J). Data in Fig. S6 are shown as mean $\pm$ SD, n=3, from one of two independent experiments. **P*<0.05, ****P*<0.001, compared with the indicated groups. n.s., no significant.

Supp. Fig. 7

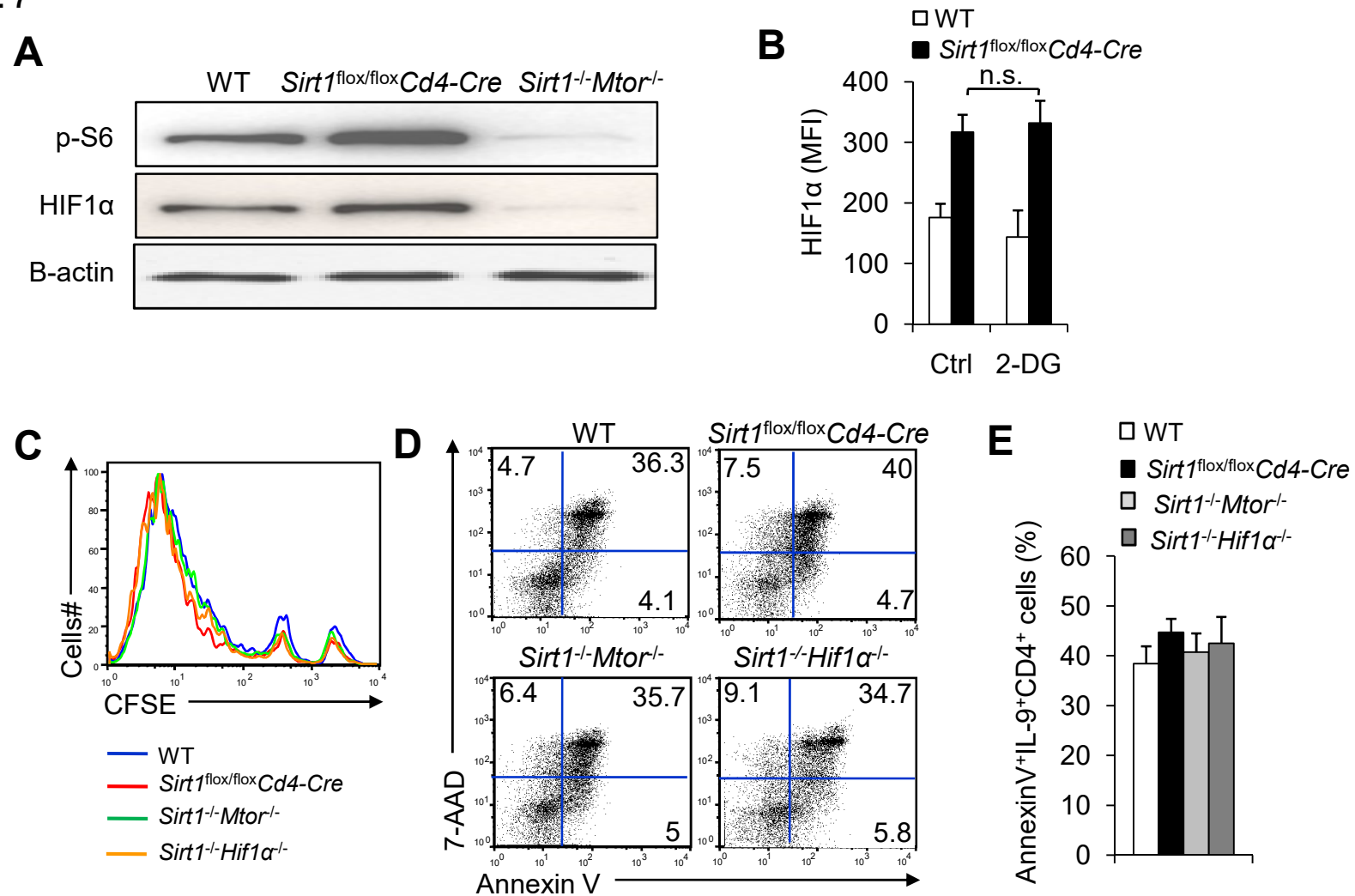


Figure S7. Th9 cell proliferation and survival. Related to Fig. 6.

(A) Immunoblot of p-S6 and HIF1α in Th9 cells. (B) Naïve T cells were differentiated under Th9 cell-inducing condition for 3 d in the presence of vehicle (Ctrl), 1 mM 2-DG. Expression of HIF1α in CD4⁺ T cells was measured and the MFI was summarized. (C-E) Naïve CD4⁺ T cells were isolated from indicated mice and were differentiated under Th9 cell conditions for 3 d. (C) Naïve CD4⁺ T cells were labeled with CFSE and CD4⁺ IL-9⁺ T cell proliferation was determined by CFSE dilution. (D-E) The frequency of apoptotic and dead CD4⁺ IL-9⁺ cells was assessed with Annexin V and 7-AAD staining. Typical figure showed in D and data summarized in E. Data in Fig. S7 are shown as mean ± SD, n=3, from one of three independent experiments. n.s., no significant.

Supplemental Experimental Procedures

Cell Purification and Culture

Lymphocytes were isolated from the spleen and lymph nodes of mice and sorted on a FACS Aria II (Becton Dickinson). The sorted naïve T cells ($CD4^{+}TCR^{+}CD62L^{high}CD44^{low}CD25^{-}$) were used for *in vitro* culture as described (Liu et al., 2010). T cells were activated with 2 ug/ml anti-CD3 (2C11; Bio X Cell), 2 ug/ml anti-CD28 (37.51; Bio X Cell) and 100 U/ml human IL-2 (R&D system). For Treg cell differentiation, cultures were supplemented with 2 ng/ml human TGF β 1 (R&D system). For Th1 cell differentiation, cultures were supplemented with 3.5 ng/ml IL-12 (R&D system) and 10 ug/ml anti-IL-4 (11B11; Bio X Cell). For Th2 cell differentiation, cultures were supplemented with 10 ng/ml IL-4 and 10 ug/ml anti-IFN- γ (all from R&D system). For Th17 cell differentiation, cultures were supplemented with 10 ug/ml anti-IL-2, 10 ug/ml IL-23, 2 ng/ml TGF β 1 and 10 ng/ml IL-6 (all from R&D system).

Flow cytometry

For FCM analysis of cell surface markers, cells were stained with antibodies in PBS containing 0.1% (wt/vol) BSA and 0.1% NaN₃ as described previously (Liu et al., 2009). The following antibodies were obtained from eBioscience: anti-CD4 (GK1.5) and anti-CD11b (M1/70). Anti-CD45 (TU116), anti-Siglec F (E50-2440), anti-F4/80 (BM8), anti-CD19 (1D3) and anti-CD11c (N418) antibodies were obtained from BD Biosciences. Anti-Histone H3 (tri methyl K4; ab8580) were obtained from Abcam. Anti-HIF1 α (IC1935P) was obtained from R&D system.

Intracellular cytokine IFN- γ (XMG1.2; BD Biosciences), IL-4 (11B11; BD Biosciences), IL-9 (D9302C12; BD Bioscience), IL-17A (TC11-18H10; BD Biosciences) staining, T cells were stimulated for 5 h with phorbol 12-myristate 13-acetate and ionomycin in the presence of monensin before being stained according to the manufacturer's instructions (BD Biosciences). Foxp3 (FJK-16s; eBioscience) and HIF1 α (IC1935P; R&D system) staining were done according to the manufacturer's instructions. For phosphorylated signaling protein staining, activated cells with IL-4 (10 ng/ml), TGF β 1 (2 ng/ml), anti-CD3 (145-2c11; BD Biosciences) and anti-CD28 (37.51; BD Biosciences), were immediately fixed with Phosflow Perm buffer (BD Biosciences), were made permeable with Phosflow Lyse/Fix buffer (BD Biosciences) and were stained with anti-p-Smad3 (C25A9; Cell Signaling Technology), anti-p-STAT6 (9361; Cell Signaling Technology), anti-p-STAT5 (C71E5; Cell Signaling Technology), anti-p-AKT (Ser 473; D9E; Cell Signaling Technology), anti-p-S6 (D57.2.2E; Cell Signaling Technology). Flow cytometry data were acquired on a FACSCalibur (Becton Dickinson, San Diego, CA) or an Epics XL bench-top flow cytometer (Beckman Coulter, CA), and data were analyzed with FlowJo

(TreeStar, San Carlos, CA). Cell numbers of various populations were calculated by multiplication of the total cell number by the percentages of each individual population from the same mouse, followed by averaging.

Retroviral transduction

SIRT1 were cloned into the MSCV retroviral vector (Clontech Laboratories, Mountain View, CA), as previously described (Liu et al., 2014). Phoenix-Eco packaging cells (ABP-RVC-10001; Allele Biotechnology, San Diego, CA) were transfected with Lipofectamine (Invitrogen), and recombinant retrovirus was collected 48 h after transfection. After 2 d of differentiation, T cells were transduced with retroviral supernatant by spin inoculation (650 x g for 1 h), as described (Liu et al., 2010). Cells were sorted based on the expression of GFP by flow cytometry and performed the cytokine production and gene-expression analysis.

Quantitative RT-PCR

Total RNA of T cells was extracted with the RNeasy kit (Qiagen, Dusseldorf, Germany), and cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). An ABI 7900 real-time PCR system was used for quantitative PCR, with primer and probe sets obtained from Applied Biosystems (Carlsbad, CA, USA). Results were analyzed using SDS 2.1 software. The expression of each target gene is presented as the 'fold change' relative to that of control samples (2-DDCT), as described previously (Liu et al., 2014).

Chromatin immunoprecipitation assay

Naïve CD4⁺ T cells isolated from spleen and/or lymph node were cultured under Th9 cell-inducing condition. Chromatin immunoprecipitation (ChIP) was performed according to the protocol described previously (Lee et al., 2015; Lim et al., 2010). Structural schematics of four predicated binding sites for HIF1 α in the *I/I9* promoter. After culture 24 h, cells were collected and then were fixed for 10 min at room temperature with 1% formaldehyde and lysed in lysis buffer (Diagenode). Lysates were sonicated, followed by precipitation with anti-HIF1 α antibodies or anti-Histone H3 antibodies for overnight. Immunoprecipitated complex were reverse-cross linked and DNA was eluted. Quantitative PCR was performed and analyzed.

Luciferase assay

Luciferase reporter assays were done as described previously (Liao et al., 2014). The mouse *I/I9* promoter region (-2410 to +32) was amplified from genomic DNA by PCR with forward primer and reverse primer. Lowercase letters in primers were Hind III and Kpn I sites was introduced to allow forced cloning into the pGL4 basic luciferase reporter gene vector (Promega). HEK293T cells were transfected with the reporter vector together with expression plasmids containing cDNA encoding mouse HIF1 α (CA-HIF1 α) and

the pRL-TK vector (Dual luciferase reporter assay system; Promega) as an internal control to exclude differences in transfection efficiency through the use of Lipofectamine 2000 method (Invitrogen). Cells were cultured for 24 h before harvesting, *IL9* promoter activity was assessed by a Dual-Luciferase reporter assay system according to the manufacturer's instructions (Promega) using a luminometer (TD20/20; Turner Designs).

SIRT1 silenced by RNA interference

A gene-silencing lentiviral construct was generated by subcloning gene-specific siRNA sequence into lentiviral small interfering RNA (siRNA) expression plasmid (pMagic4.1; Sbo-bio, Shanghai, China). SIRT1 siRNA sequence is 5'-CAGGTTGCAGGAATCCAAAG-3' was synthesized (Takala Co. Ltd., Dalian, China), as described previously (Liu et al., 2014). Lentiviruses were harvested from culture supernatant of 293T cells (CL1032; Abgent, San Diego, CA) transfected with siRNA vector. Sorted naïve T cells from mice or human were stimulated for 24 h with anti-CD3 and anti-CD28 (both identical above) and were infected with recombinant lentivirus. After overnight culture, cells were restimulated for 24 h with TGFβ1 and IL-4, followed by real-time PCR analysis of the target mRNA or IL-9 mRNA.

Gene expression profiling by microarray analysis

RNA samples collected from biological triplicates were analyzed using the agilent feature extraction software (version 11.0.1.1) and expression signals were summarized using GeneSpring GX v12.1 software package (Agilent Technologies). Differentially expressed genes were identified through Fold Change filtering. Hierarchical Clustering was performed using the R scripts. The entire microarray data was deposited into the GEO series database under accession number GSE79607.

Glycolysis flux assay

Glycolysis of T cells were determined by measuring the detritiation of [3-³H]-glucose (PerkinElmer) and, 2 h later, the medium was transferred to microcentrifuge tubes containing 50 ul 5N HCL. The microcentrifuge tubes were then placed in 20 ml scintillation vials containing 0.5 ml water, and the virals were capped and sealed. ³H₂O was separated from unmetabolized [3-³H]-glucose by evaporation diffusion for 24 h at room temperature, as described previously (Liu et al., 2014).

Human T cells *in vitro*

Human peripheral blood CD4⁺ T cells (6902K-50a; Cell applications) were purified with a naïve T cell isolation kit (Miltenyi Biotec). Naïve T cells were cultured for 48 h with plate-bound anti-human CD3 (1 ug/ml) and soluble anti-human CD28 (1 ug/ml) with TGFβ1 (2 ng/ml) plus IL-4 (10 ng/ml). Optimized dose of Ex527 (10 μM/L), SIRT1720 (10 μM/L), Rapamycin (50 nM/L), 2-ME (2 μM/L) and 2-DG (1 mmol/L) were used in human T cell culture.

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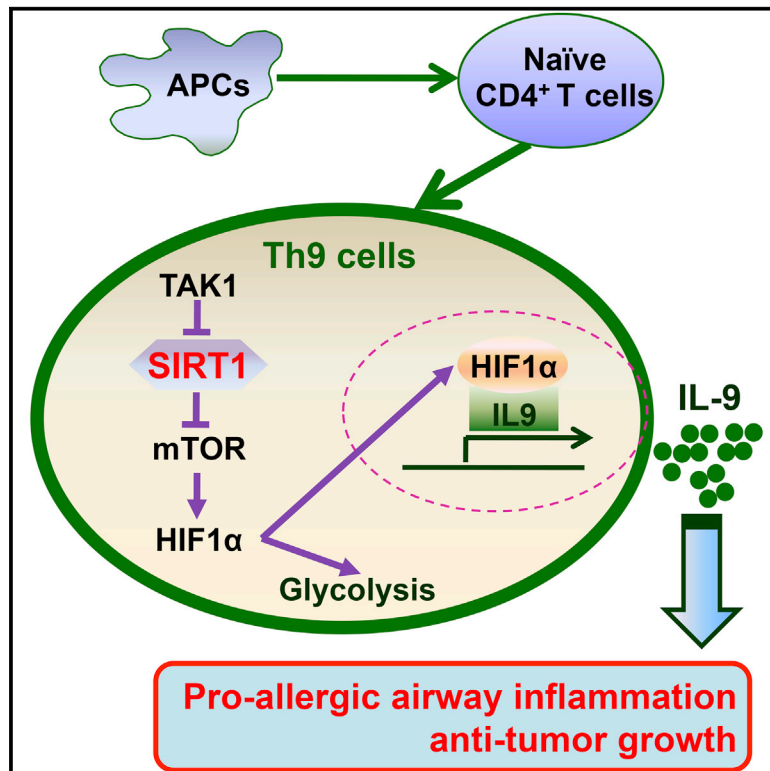
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Histone Deacetylase SIRT1 Negatively Regulates the Differentiation of Interleukin-9-Producing CD4⁺ T Cells

Graphical Abstract



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In Brief

Th9 cells are critical in controlling immune-associated diseases. Liu and colleagues demonstrate that histone deacetylase SIRT1 negatively regulates the activities of mTOR and HIF1 α signaling, couples with glycolysis metabolism, suppresses IL-9 production of CD4⁺ T cells, alleviates allergic airway inflammation, and promotes cancerous tumor growth.

Highlights

- Deficiency of SIRT1 in CD4⁺ T cells increased IL-9 production and glycolysis
- Ectopic SIRT1 expression in CD4⁺ T cells inhibited IL-9 production and glycolysis
- SIRT1-dependent Th9 cells regulated tumor and allergic pulmonary inflammation
- TAK1-SIRT1-mTOR-HIF1 α -glycolysis pathway was required for Th9 cell differentiation

Accession Numbers

GSE79607



Histone Deacetylase SIRT1 Negatively Regulates the Differentiation of Interleukin-9-Producing CD4⁺ T Cells

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SUMMARY

Distinct metabolic programs support the differentiation of CD4⁺ T cells into separate functional subsets. In this study, we investigated metabolic mechanisms underlying the differentiation of IL-9-producing CD4⁺ T cells (Th9) in allergic airway inflammation and cancerous tumors. We found that histone deacetylase SIRT1 negatively regulated Th9 cell differentiation. A deficiency of SIRT1 induced by either conditional deletion in mouse CD4⁺ T cells or the use of small interfering RNA (siRNA) in mouse or human T cells increased IL-9 production, whereas ectopic SIRT1 expression inhibited it. Notably, SIRT1 inhibited Th9 cell differentiation that regulated anti-tumor immunity and allergic pulmonary inflammation. Glycolytic activation through the mTOR-hypoxia-inducible factor-1 α (HIF1 α) was required for the differentiation of Th9 cells that conferred protection against tumors and is involved in allergic airway inflammation. Our results define the essential features of SIRT1-mTOR-HIF1 α signaling-coupled glycolytic pathway in inducing Th9 cell differentiation, with implications for metabolic reprogramming as an immunotherapeutic approach.

INTRODUCTION

The engagement of a variety of specific metabolic pathways profoundly affects T cell differentiation and function (Man and Kalles, 2015). Therefore, it is no surprise that distinct metabolic programs also support the differentiation of CD4⁺ T helper (Th) cells into their separate functional subsets (Zeng and Chi, 2015). It has been demonstrated that Th1, Th2, and Th17 cells are strongly dependent on glycolysis, whereas regulatory T (Treg) cells depend more on the oxidation of lipids (Waickman and Powell, 2012). However, the specific modulation of metabolism that is involved in Th9 cell differentiation remains unclear.

SIRT1 is the highly conserved mammalian NAD⁺-dependent histone deacetylase and has emerged as a key metabolic sensor in various metabolic tissues (Yang et al., 2015). In response to different environmental stimuli, SIRT1 directly links to the cellular metabolism of gene expression, thereby modulating a variety of cellular processes, such as energy metabolism and the stress response (Lim et al., 2010; Yang et al., 2015). SIRT1 is also a key transcriptional regulator of inflammation. In innate immune cells, SIRT1 inhibits the production of proinflammatory cytokines in macrophages and dendritic cells (DCs) (Yang et al., 2015). In myeloid-derived suppressor cells (MDSCs), SIRT1 directs the differentiation of MDSCs in anti-tumor immunity (Liu et al., 2014). Furthermore, DC SIRT1 has been reported to direct T cell differentiation from Th1 to Treg cells in the course of microbial-associated infection (Liu et al., 2015) and to promote Th2 cell differentiation in airway allergy (Legutko et al., 2011). In addition, SIRT1 is required to maintain T cell tolerance (Yang et al., 2015). However, whether SIRT1 is involved in the metabolism-dependent modulation of the differentiation of intrinsic T cells remains unclear.

In the present study, we have demonstrated that SIRT1-dependent glycolytic metabolism modulation is critical for directing the differentiation of Th9 cells under physiological conditions as well as pathological conditions such as Th9 cell-associated allergic airway inflammation and tumors. Directly targeting the *Il9* gene locus, SIRT1 inhibits the differentiation of Th9 cells by an mTOR-HIF1 α -dependent signaling coupled with glycolytic pathway.

RESULTS

Th9 Cell Differentiation Is Associated with SIRT1-Dependent Glycolytic Activity

Although metabolic signaling regulates T cell differentiation, the mechanism of glycolytic metabolism in different T cell subsets remains unclear. We therefore investigated the glycolytic activities in various T cell subsets. As previously reported (Liu et al., 2010), Th1, Th2, Th17, Treg, and Th9 cells could be induced in vitro and obtained (Figure 1A, left). The glycolytic activity of each T cell subset was measured by the generation of ³H-labeled H₂O from [3-³H]-glycose. Consistent with a previous report, Th1,

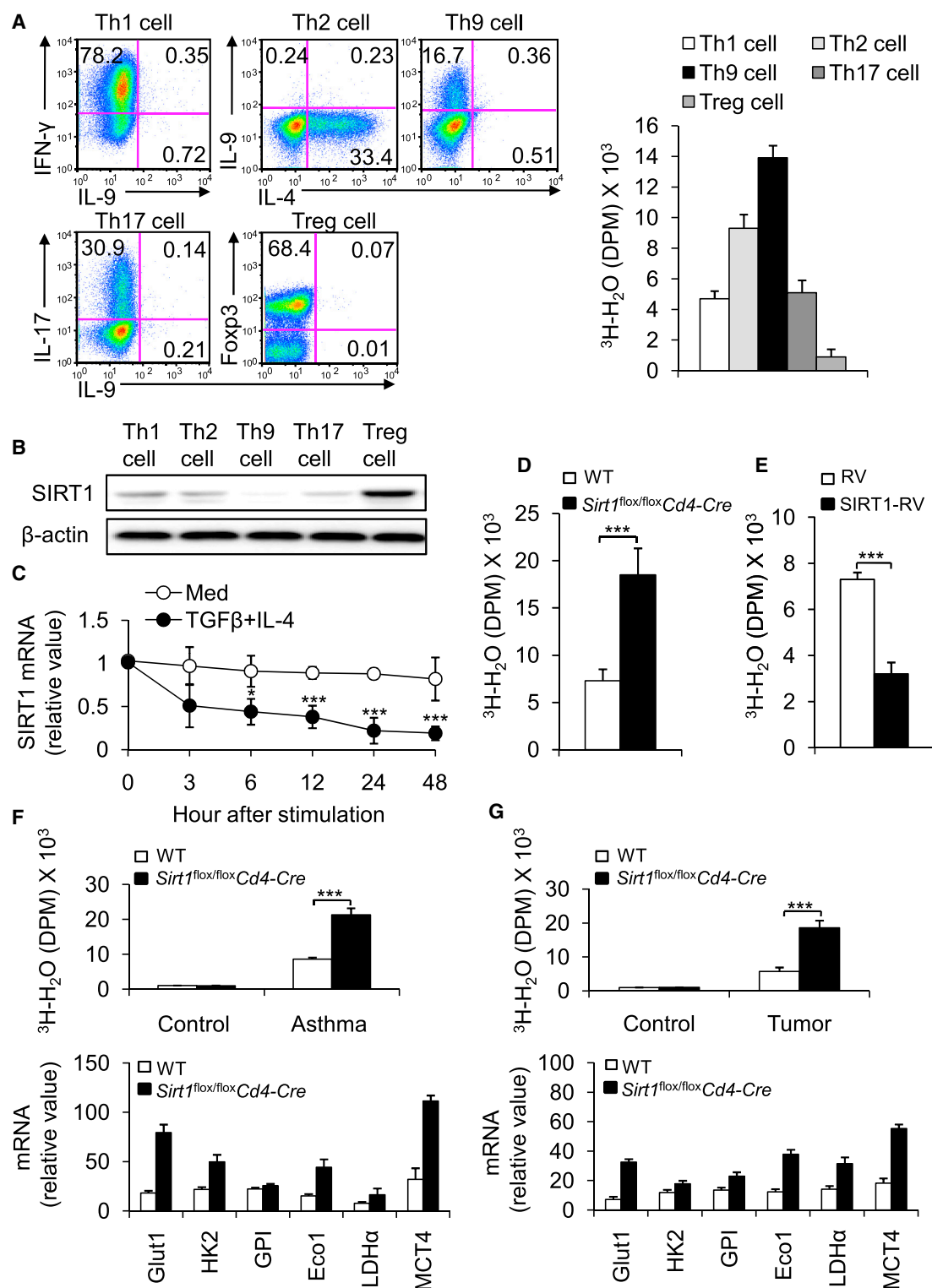


Figure 1. The Th9 Cell-Inducing Condition and Th9 Cell-Associated Allergic Airway Inflammation and Tumors along with Alterations in SIRT1-Dependent Glycolytic Activity

(A) Sorted naive T cells ($\text{CD4}^+\text{TCR}^+\text{CD62L}^{\text{hi}}\text{CD44}^{\text{lo}}\text{CD25}^-$) from WT mice were differentiated under Th1, Th2, Th9, Th17, or Treg cell-inducing conditions for 5 days, followed by intracellular staining of IFN- γ (Th1 cells), IL-4 (Th2 cells), IL-17 (Th17 cells) or Foxp3 (Treg cells), and IL-9 (left). The glycolytic activity of these cells was measured by the generation of ^3H -labeled H_2O from [^3H]-glycose (right).

(B) SIRT1 expression was examined by western blot in T cells activated under various conditions for 5 days.

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Th2, and Th17 cells exhibited much higher while Treg cells exhibited less glycolytic activity (Shi et al., 2011). Moreover, Th9 cells exhibited more glycolytic activity than Th1, Th2, Th17, or Treg cells (Figure 1A, right). These results suggest that Th9 cell differentiation is probably related to glycolytic metabolism.

The key metabolic regulator SIRT1 that has previously been shown to target dendritic cells (DCs) regulates the differentiation of Th1 and Treg cells (Liu et al., 2015). We investigated whether SIRT1 regulates the glycolytic activity of T cell subsets. SIRT1 was found to be differentially induced in all of the T cell subsets (Figures 1B and S1A). Treg cells exhibited the highest expression, whereas Th9 cells exhibited a lower expression than Th1, Th2, and Th17 cells (Figure 1B). Consistent with this result, a Th9 cell-inducing condition (IL-4 plus TGF- β 1) resulted in a decrease of SIRT1 mRNA (Figures 1C and S1A). IL-4 alone did not affect the expression of SIRT1, but TGF- β 1 plus IL-4 caused the most significant downregulation of SIRT1 (Figure S1B). This result suggests that the optimized Th9 cell-polarizing cytokine milieu is critical for suppressing SIRT1. We next studied the molecular mechanisms underlying the TGF- β 1-mediated downregulation of SIRT1 in Th9 cells. We found that Smad3, an inhibitor of transcriptional factor Smad3, an important mediator of a canonical TGF- β 1 signaling, did not alter the SIRT1 expression, although it did suppress the expression of IL-9 (Figures S1C and S1D). This result suggested that the Smad-dependent pathway is not involved in regulating SIRT1 in Th9 cell differentiation. The TGF- β -triggered kinase TAK1 is an important mediator of the TGF- β signaling that is independent of Smad (Choi et al., 2012). TAK1 has also been reported as a key player in directing Th9 cell differentiation (Nakatsukasa et al., 2015). We therefore investigated the role of TAK1 in regulating SIRT1 expression during Th9 cell differentiation. We found that 5z-7-oxozeanin, a potent TAK1 inhibitor, completely blocked IL-9 expression but did not substantially change the differentiation of Th1, Th2, Th17, or Treg cells (Figure S1E). These data confirmed the critical role of TAK1 in Th9 cell differentiation as previously reported. Importantly, the inhibition of TAK1 significantly reversed the suppression of SIRT1 (Figure S1F), which suggested that Smad-independent TAK1 signal was responsible for the suppression of SIRT1 during Th9 cell differentiation. Consistent with the pharmacologic result, the TAK1 genetic silencing suppressed IL-9 production and reversed the downregulation of SIRT1 expression during Th9 cell differentiation (Figures S1G–S1I). Together, these data suggest that the TAK1 selectively suppresses SIRT1 during Th9 cell differentiation.

To test the role of SIRT1 in Th9 cell differentiation, we crossed *Sirt1*^{flox/flox} and *Cd4-Cre* mice to conditionally delete *Sirt1* gene expression in T cells (called *Sirt1*^{flox/flox}*Cd4-Cre* mice hereafter). Immunoblot and real-time PCR results showed that SIRT1 was specifically deleted in T cells in this mouse (Figures S1J and S1K). Thus, we observed the glycolytic activity of Th9 cells obtained from *Sirt1*^{flox/flox}*Cd4-Cre* mice. Sorted naive T cells (CD4⁺TCR⁺CD62L^{hi}CD44^{lo}CD25[−] cells) from both the wild-type (WT) and *Sirt1*^{flox/flox}*Cd4-Cre* mice were differentiated under Th9 or other T cell subset-inducing conditions, and the glycolytic activity was measured as described above. SIRT1 deficiency increased the glycolytic activity in Th9 cells (Figures 1D and S2A). This suggested that SIRT1 is required for the glycolytic modulation of Th9 cells. To test whether SIRT1 is sufficient for the glycolytic activity of Th9 or other T cell subsets, we ectopically expressed SIRT1 with retrovirus under various T cell subset-inducing conditions. Modest SIRT1 overexpression was shown to inhibit Th9 cell glycolytic activity (Figures 1E and S2B). Thus, these data demonstrate that SIRT1 is involved in the glycolytic modulation of Th9 cell differentiation.

To ascertain the role of SIRT1-dependent glycolytic activity in regulating Th9 cell differentiation, we directly compared Th9 cells from Th9-associated diseases. We subjected WT and *Sirt1*^{flox/flox}*Cd4-Cre* mice to a standard protocol for the development of ovalbumin (OVA)-induced allergic airway inflammation in which Th9 cells have been shown to be involved (Wilhelm et al., 2011). We sensitized mice with OVA in aluminum hydroxide and challenged them intranasally with OVA in PBS. CD4⁺ T cells were isolated from lung-local draining lymph nodes (DLNs). As expected, there were markedly reduced SIRT1 mRNA and increased IL-9 mRNA expression in OVA-sensitized asthma compared with control cells (Figures S2C and S2D). These results indicate that SIRT1 downregulation is probably negatively related in IL-9-dependent allergic airway inflammation. Furthermore, the glycolytic activity of T cells was measured in both WT and *Sirt1*^{flox/flox}*Cd4-Cre* mice in OVA-sensitized asthma. *Sirt1*^{flox/flox}*Cd4-Cre* mice exhibited much more glycolytic activity than WT mice (Figure 1F, top). These findings suggest that SIRT1-dependent glycolytic activity is associated with Th9 cell differentiation in allergic airway inflammation.

Recently, Th9 cells have been reported to inhibit tumor growth (Purwar et al., 2012). To directly observe the role of Th9 cells in a tumor model, we used a T cell adoptive transfer system. Isolated naive T cells from WT or *Sirt1*^{flox/flox}*Cd4-Cre* mice were differentiated under Th9 cell-inducing conditions and the same number

(C) SIRT1 mRNA expression as examined by real-time PCR analysis in T cells activated by Th9 cell-inducing conditions for different time courses. Expressions in naive cells were set to 1.

(D) Naive T cells from WT and *Sirt1*^{flox/flox}*Cd4-Cre* mice were differentiated under Th9 cell conditions, followed by the measurement of glycolytic activity.

(E) eGFP⁺ cells sorted from the Th9 cell condition that were transduced with control retrovirus (RV) or *Sirt1*-expressing retrovirus (SIRT1-RV) were measured for glycolytic activity.

(F) WT and *Sirt1*^{flox/flox}*Cd4-Cre* mice were sensitized and challenged intranasally with OVA; T cells were isolated from pulmonary DLNs at 48 hr after the final intranasal challenge. The glycolytic activity of these cells was measured by the generation of ³H-labeled H₂O from [3-³H]-glucose (top). RNA was isolated from DLN T cells in WT or *Sirt1*^{flox/flox}*Cd4-Cre* mice and stimulated for 72 hr with anti-CD3, then used for RT-PCR analysis of glycolytic molecules (bottom).

(G) *Rag1*^{−/−} mice were given intravenous transfer of WT or *Sirt1*^{flox/flox}*Cd4-Cre* naive CD4⁺ T cells (stimulated via anti-CD3 and anti-CD28, together with TGF- β 1 and IL-4 for 3 days) along with simultaneous subcutaneous injection of B16 melanoma cells on day 0. After 30 days, CD4⁺ T cells were isolated from mouse solid tumors in WT or *Sirt1*^{flox/flox}*Cd4-Cre* mice and the glycolytic activity of these cells was measured by the generation of ³H-labeled H₂O from [3-³H]-glycose. All tumor tissues were subjected to the same enzymatic digestion (top). RNA was isolated from tumor-derived T cells in WT or *Sirt1*^{flox/flox}*Cd4-Cre* mice and these cells were stimulated for 72 hr with anti-CD3, and then used for RT-PCR analysis of glycolytic molecules (bottom).

Data are representative of three to four independent experiments (n = 4). *p < 0.05; ***p < 0.001, compared with the indicated groups. See also Figures S1 and S3.

of cells intravenously injected into *Rag1*^{-/-} mice, which have a congenital deficiency in mature B cells and T cells. Then we subcutaneously injected B16 melanoma cells into the *Rag1*^{-/-} recipient mice. On day 20 the mice received CD4⁺ T cells, the tumor tissue was digested, and the CD4⁺ T cells were isolated. As expected, SIRT1 mRNA expression was markedly reduced whereas IL-9 mRNA expression in tumors was increased compared with control cells (Figures S2E and S2F). These data suggest that SIRT1 downregulation is negatively related to IL-9 production in tumors. Furthermore, *Sirt1*^{flox/flox}*Cd4-Cre* T cells exhibited much more glycolytic activity than WT control (Figure 1G, top). This finding indicates that SIRT1-dependent glycolytic activity is probably associated with Th9 cell differentiation in tumors.

There are multiple glucose-associated enzymes that play an important role in controlling the generation of lactate and ATP molecules as energy sources. Consistent with this, transcriptional mRNA analyses of Glut1, glucose-6-phosphate isomerase, Eno1 (enolase 1), LDH α , and monocarboxylic acid transporter member 4 in WT T cells isolated from lung DLNs in asthma (Figure 1F, bottom) or a tumor (Figure 1G, bottom) revealed marked upregulation of genes encoding various molecules involved in glycolysis. Taken together, the SIRT1-dependent glycolytic activities associated with Th9 cell differentiation.

SIRT1 Inhibits Th9 Cell Differentiation

To clarify the role of SIRT1 in Th9 cell differentiation, we ectopically expressed SIRT1 with a retrovirus and sorted the positive green fluorescent protein (GFP) T cells, observing the alteration in IL-9 expression that took place under various T cell differentiation-inducing conditions, as described above. Modest SIRT1 overexpression (SIRT1-RV) resulted in a decrease in IL-9 mRNA expression, IL-9 secretion, and the ratio of IL-9⁺CD4⁺ T cells under Th9 cell-inducing conditions, but not Th1, Th2, Th17, or Treg cell-inducing conditions, compared with control (RV; Figures 2A–2C). In addition, even IL-4 expression was not altered in the SIRT1-RV group under either Th0 or Th9 cell-inducing conditions (Figure 2C). Moreover, IL-9 mRNA expression and IL-9 secretion exhibited potent and sustained downregulation in SIRT1-RV compared with RV (Figures 2D and 2E). Importantly, consistent with the alteration in glycolytic activity (Figure 1E), the glucose-associated enzyme molecules were diminished in SIRT1-RV compared with RV (Figure 2F), indicating that the SIRT1-dependent glycolytic is sufficient to regulate Th9 cell differentiation.

SIRT1 Deficiency Enhances Th9 Cell Differentiation

To ascertain the role of SIRT1 in Th9 cell differentiation, we obtained differentiated naive T cells from WT and *Sirt1*^{flox/flox}*Cd4-Cre* mice under various T cell subset-inducing conditions, as described above. We found that IL-9 mRNA expression, IL-9 secretion, and the IL-9⁺ cell ratio in T cells from *Sirt1*^{flox/flox}*Cd4-Cre* were higher than the WT under Th9 cell-inducing conditions (Figures 3A–3C). Naive WT CD4⁺ T cells did not produce IL-9 after being cultured with either TGF- β 1 or IL-4 alone. However, naive *Sirt1*^{flox/flox}*Cd4-Cre* T cells readily produced a substantial amount of IL-9 in response to either TGF- β 1 or TGF- β 1 plus IL-4 under Th9 cell-inducing conditions in an IL-4 dose-dependent manner (Figures 3C and S2G). Moreover, IL-9

mRNA expression and IL-9 secretion exhibited strong and sustained upregulation in *Sirt1*^{flox/flox}*Cd4-Cre* compared with the WT (Figures 3D and 3E). Importantly, consistent with the glycolytic activity (Figure 1D), the glucose-associated enzyme molecules were significantly increased in *Sirt1*^{flox/flox}*Cd4-Cre* compared with the WT (Figures S2H and S2I). Notably, we also acutely deleted SIRT1 in naive WT CD4⁺ T cells using siRNA. SIRT1 efficient silencing resulted in the upregulation of IL-9 mRNA expression and a much higher IL-9⁺ cell ratio in CD4⁺ T cells compared with control (Figures 3F and 3G). Consistent with this, the glycolytic activity was significantly increased in SIRT1 siRNA compared with control cells (Figure S2J), indicating that SIRT1-dependent glycolytic activity is required for directing Th9 cell differentiation.

To understand how SIRT1 regulates Th9 cell differentiation, we compared the gene expression profiles of WT and *Sirt1*^{flox/flox}*Cd4-Cre* cells differentiated toward Th9 cells using Affymetrix oligonucleotide arrays and found that the expression of a selective groups of metabolic molecules, including glycolytic enzymes described in Figures 1F and 1G, were upregulated in *Sirt1*^{flox/flox}*Cd4-Cre* cells. *Sirt1*^{flox/flox}*Cd4-Cre* cells also displayed altered expression patterns of transcription factors, signaling molecules, and surface or secreted factors, including Hif1 α , and, as expected, IL-9 (Figure 3H). These data demonstrate that diverse SIRT1 target genes in T cells regulate T cell differentiation and metabolism.

The IL-9 Produced by SIRT1 Deficiency CD4⁺ T Cells Is Involved in the Development of Allergic Airway Inflammation

It has been reported that IL-9 is involved in allergic airway inflammation (Wilhelm et al., 2011). To investigate the role of SIRT1-dependent Th9 cell differentiation, we set up an OVA-induced allergic airway inflammation model in WT and *Sirt1*^{flox/flox}*Cd4-Cre* mice, as described above. *Sirt1*^{flox/flox}*Cd4-Cre* mice exhibited significantly more severely pathogenic lung inflammation and greater inflammatory cell recruitment, including eosinophil infiltration in bronchoalveolar lavage fluid (BALF), compared with the WT (Figures 4A, 4B, S3A, and S3B). The CD4⁺ T cells isolated from the lung DLNs of *Sirt1*^{flox/flox}*Cd4-Cre* model mice exhibited a greater number of IL-9⁺ cells, but not IFN- γ ⁺, IL-4⁺, IL-17⁺, or Foxp3⁺ cells, compared with WT (Figures 4C, S3C, and S3D). Moreover, SIRT1 deficiency resulted in higher IL-9 secretion (Figure 4D) accompanied by higher glycolytic activity (Figure S3E). The proliferation of antigen-specific T cells, however, was similar (Figure S3F). Importantly, blocking IL-9 with an anti-IL-9 antibody markedly restored all these pathological alterations (Figures 4A–4D and S3A–S3D). Taken together, the data show that SIRT1 is required for suppressing Th9 cell differentiation in alleviating Th9 cell-associated allergic airway inflammation.

To directly test the role of SIRT1 in Th9 cell-dependent diseases, we used an adoptive transfer model. Naive T cells isolated from WT or *Sirt1*^{flox/flox}*Cd4-Cre* mice were differentiated under Th9 cell-inducing conditions and transferred into *Rag1*^{-/-} mice, which were sensitized and challenged with OVA on day 0. The mice that received *Sirt1*^{flox/flox}*Cd4-Cre* CD4⁺ T cells displayed severe pathological lung damage and eosinophil infiltration in BALF compared with the WT (Figures S4A and S4B).

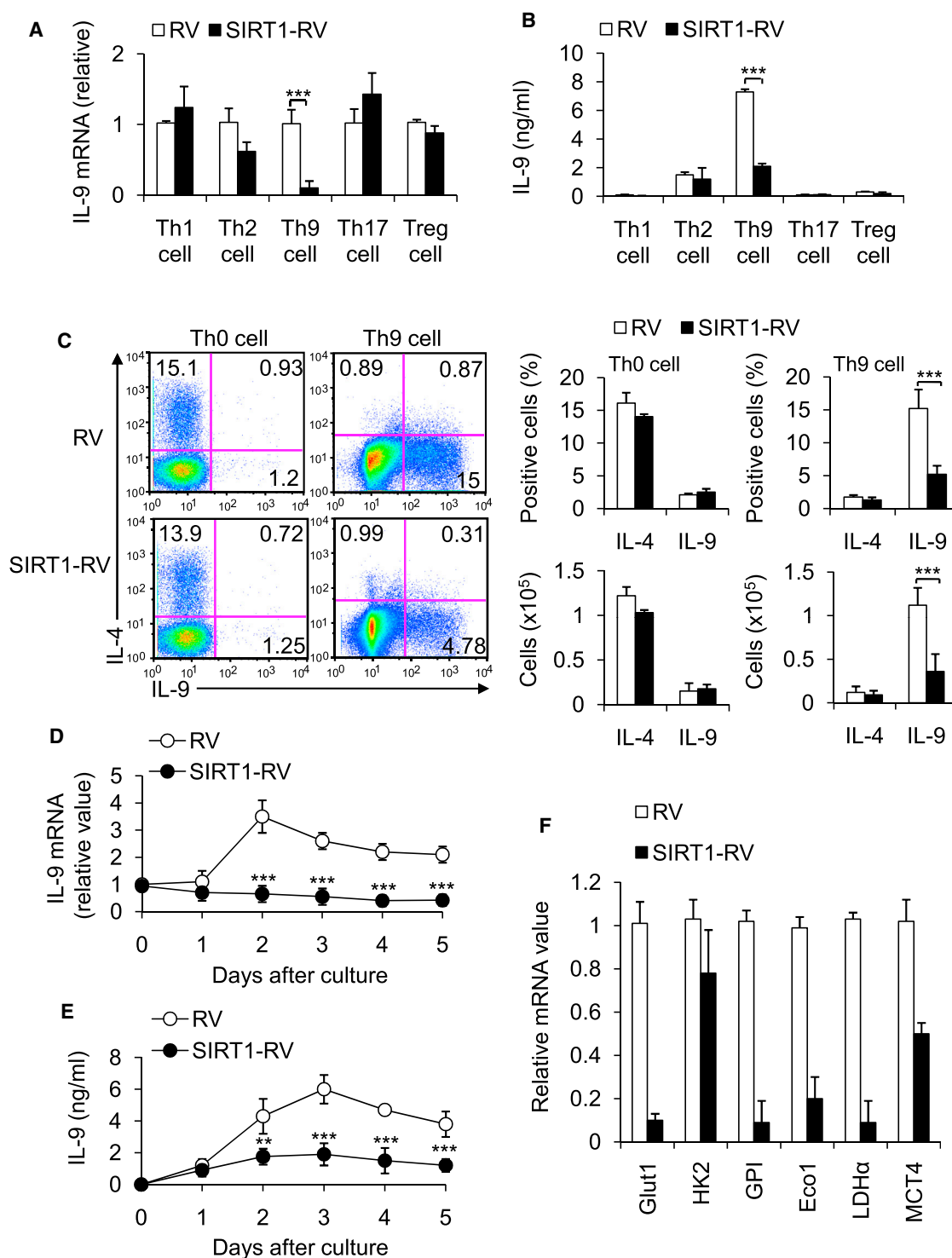


Figure 2. SIRT1 Inhibits Th9 Cell Differentiation

(A and B) IL-9 expression (A) and IL-9 secretion (B) by eGFP⁺ cells sorted from the indicated cell-inducing conditions were transduced with control retrovirus (RV) or SIRT1-expressing retrovirus (SIRT1-RV) and stimulated for 24 hr with anti-CD3. Expressions in the RV groups were set to 1.

(C) Intracellular staining of IL-4 and IL-9 in the eGFP⁺ populations from the Th9 or Th0 cell-inducing conditions transduced with RV or SIRT1-RV.

(D and E) The time course changes in IL-9 mRNA expression (D) and IL-9 secretion (E) by eGFP⁺ cells from the Th9 cell conditions transduced with RV or SIRT1-RV. (F) Glycolytic molecule expressions by eGFP⁺ cells sorted from the Th9 cell-inducing conditions transduced with RV or SIRT1-RV and stimulated for 24 hr with anti-CD3, then used for RT-PCR analyses of glycolytic molecules (expressions in the RV groups were set to 1).

Data are representative of three to four independent experiments (n = 4). **p < 0.01; ***p < 0.001, compared with the indicated groups.

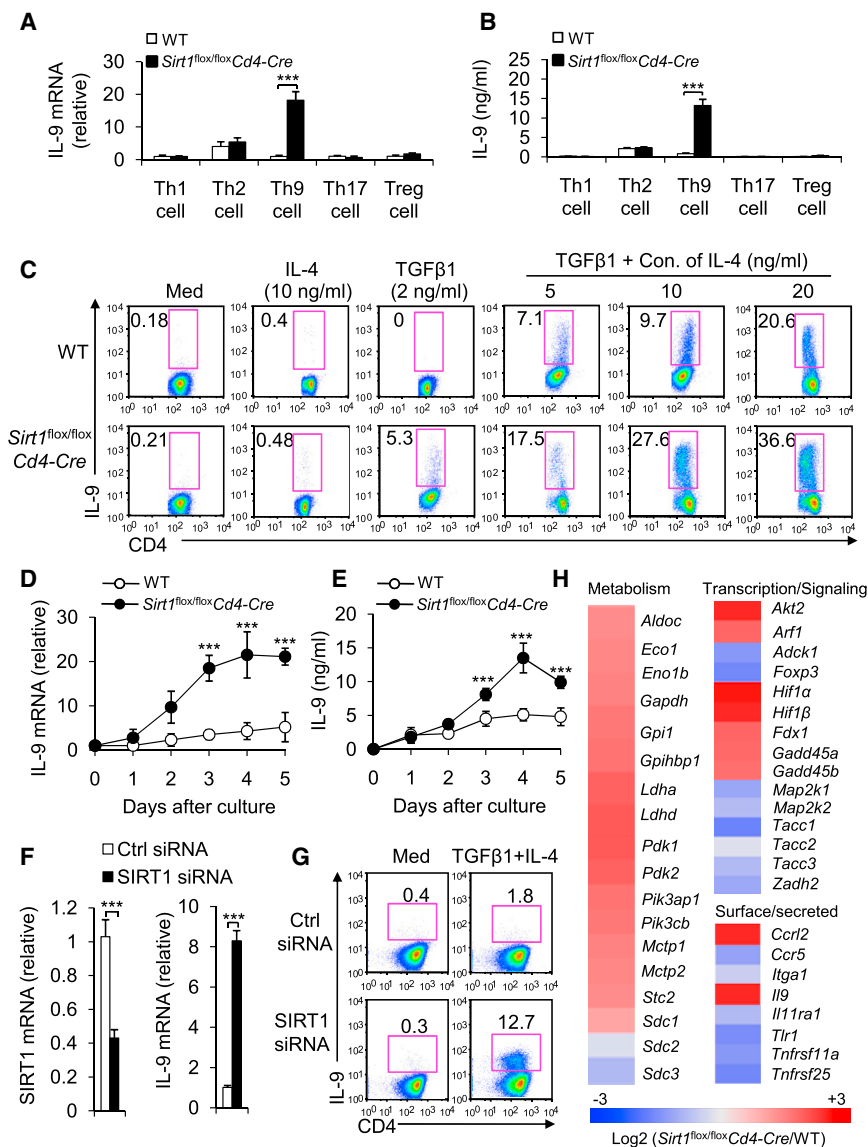


Figure 3. SIRT1 Deficiency Enhances Th9 Cell Differentiation

(A and B) IL-9 expression (A) and IL-9 secretion (B) by CD4⁺ T cells sorted from the indicated cell-inducing conditions in WT or *Sirt1*^{fllox/fllox} *Cd4-Cre* mice and stimulated for 24 hr with anti-CD3. Expressions in WT naive T cells were set to 1.

(C) Intracellular staining of IL-9 in WT and *Sirt1*^{fllox/fllox} *Cd4-Cre* CD4⁺ T cells cultured for 72 hr with anti-CD3 and anti-CD28, plus medium alone or the various indicated combinations.

(D and E) The time course changes in IL-9 expression (D) and secretion (E) by CD4⁺ cells under Th9 cell-inducing conditions in WT and *Sirt1*^{fllox/fllox} *Cd4-Cre* mice. Expressions in WT naive T cells were set to 1.

(F) Expression of SIRT1 (left) and IL-9 mRNA (right) in naive CD4⁺ T cells from WT mice transfected with control siRNA (Ctrl siRNA), or SIRT1-specific siRNA (SIRT1 siRNA), and stimulated for 48 hr with anti-CD3 and anti-CD28 plus TGF-β1 and IL-4 (expressions in Ctrl siRNA were set to 1).

(G) Intracellular IL-9 expression in cells transfected as in (F) and stimulated with anti-CD3 and anti-CD28 plus medium (med) alone or TGF-β1 and IL-4.

(H) Naive T cells from WT and *Sirt1*^{fllox/fllox} *Cd4-Cre* mice were differentiated under Th9 cell-inducing condition for 3 days, and then RNA was analyzed by microarrays to compare the expression profiles of the WT and *Sirt1*^{fllox/fllox} *Cd4-Cre* cells with certain genes involved in metabolism, transcription factors, or signaling pathways, and surface or secreted molecules. Heat maps display gene expression relative to the WT mean on a log2 scale.

Data are representative of three to four independent experiments (A–G; n = 3–5). ***p < 0.001, compared with the indicated groups. See also Figures S1 and S3.

Infiltrating CD4⁺ T cells isolated from the lung DLNs of the *Sirt1*^{fllox/fllox} *Cd4-Cre* group were stimulated with anti-CD3 for 24 hr and exhibited higher IL-9 expression and glycolytic activity compared with the control (Figures S4C and S4D). However, blocking IL-9 restored all these pathological alterations (Figure S4). Thus, these data indicate that SIRT1 is required for Th9 cell-dependent allergic airway inflammation.

The IL-9 Produced by SIRT1-Deficient CD4⁺ T Cells Is Involved in Delaying Tumor Growth

Recently, Th9 cells have been reported to inhibit tumor growth (Purwar et al., 2012). To directly investigate the role of SIRT1-dependent Th9 cell differentiation, we set up a tumor model in WT and *Sirt1*^{fllox/fllox} *Cd4-Cre* mice, as described above. Naive T cells isolated from WT or *Sirt1*^{fllox/fllox} *Cd4-Cre* mice were differentiated under Th9 cell-inducing conditions and transferred into *Rag1*^{−/−} mice, and then we subcutaneously injected these mice with B16 melanoma cells. The mice that received

Sirt1^{fllox/fllox} *Cd4-Cre* CD4⁺ T cells exhibited tumors of smaller size compared with the WT control (Figure 4E). Tumor-infiltrating CD4⁺ T cells isolated from the *Sirt1*^{fllox/fllox} *Cd4-Cre* groups displayed higher IL-9 expression, IL-9 secretion, and IL-9⁺ ratio accompanied by higher glycolytic activity, compared with WT (Figures 4F–4H and S5). Importantly, blocking IL-9 with an anti-IL-9 antibody restored the *Sirt1*^{fllox/fllox} *Cd4-Cre* Th9 cell transfer-elicited changes, except for the glycolytic activity (Figures 4E–4H and S5). Taken together, these data show that SIRT1 is required for suppressing Th9 cell differentiation in inhibiting Th9 cell-dependent anti-tumor immunity.

Blocking the Glycolytic Metabolism Pathway Restores SIRT1-Deficiency-Induced Th9 Cell Differentiation

To investigate the role of SIRT1-dependent glycolytic activity in Th9 cell differentiation, naive T cells were differentiated under Th9 cell-inducing conditions in the presence or absence of 2-DG, a prototypical inhibitor of the glycolytic pathway that

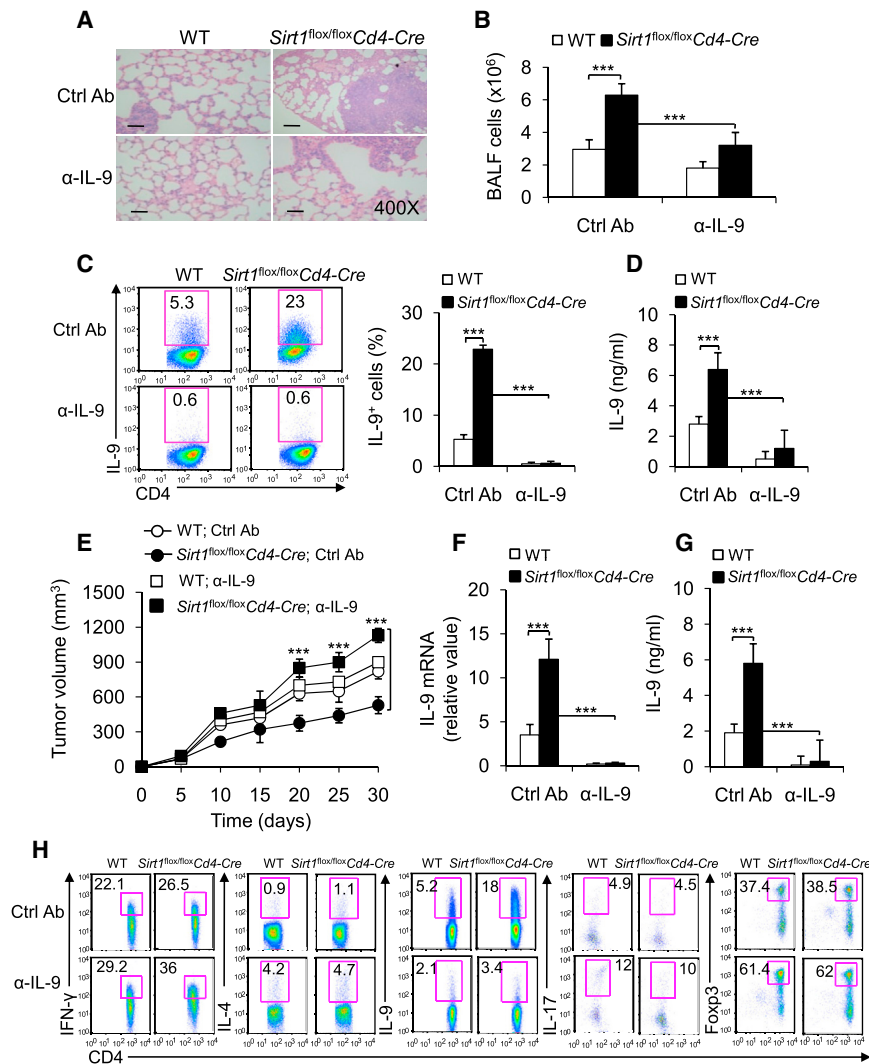


Figure 4. IL-9 Produced by SIRT1-Deficient CD4⁺ T Cells Is Involved in Allergic Airway Inflammation and Anti-tumor Immunity

(A–D) WT and *Sirt1*^{fllox/fllox}*Cd4-Cre* mice were sensitized and challenged intranasally with OVA on day 0, then treated with anti-IL-9 (α -IL-9) or isotype-matched control antibody (Ctrl Ab) every 3 days from day 0.

(A) Hematoxylin and eosin staining of paraffin-embedded sections of lungs from mice challenged as described above 48 hr after the final OVA challenge. Original magnification, $\times 400$.

(B) The absolute total cell number of BALF. (C) Intracellular IL-9 expression of CD4⁺ T cells isolated from lung DLNs of WT and *Sirt1*^{fllox/fllox}*Cd4-Cre* mice, with a typical figure shown (left) and the frequencies of positive cells are summarized (right).

(D) Secretion of IL-9 of CD4⁺ T cells isolated from the BALF of mice challenged as described above was measured with ELISA.

(E–H) *Rag1*^{-/-} mice given intravenous transfer of WT or *Sirt1*^{fllox/fllox}*Cd4-Cre* CD4⁺ T cells (stimulated via anti-CD3 and anti-CD28, together with TGF- β 1 and IL-4 for 3 days) along with simultaneous subcutaneous injection of B16 melanoma cells on day 0, then treated with anti-IL-9 (α -IL-9) or isotype-matched control antibody (Ctrl Ab) every 3 days from day 0.

(E) The tumor growth curve was plotted. (F and G) Expression (F) or secretion (G) of IL-9 by CD4⁺ T cells sorted from the indicated mouse tumor-infiltrating cells and stimulated for 24 hr with anti-CD3. Expressions are presented relative to those that control gene *Gapdh*.

(H) Intracellular IFN- γ , IL-4, IL-17, and Foxp3 expression of CD4⁺ T cells isolated from tumor-infiltrating cells in WT and *Sirt1*^{fllox/fllox}*Cd4-Cre* mice, with a typical figure shown.

Data are representative of three independent experiments ($n = 3–10$). *** $p < 0.001$, compared with the indicated groups. See also Figures S3–S5.

acts via blocking hexokinase, the first rate-limiting enzyme of glycolysis, and the presence or absence of rapamycin, an mTOR inhibitor, which can block the mTOR-dependent metabolic pathway, including glycolysis, as a control. As expected, optimized doses of 2-DG or rapamycin significantly diminished the glycolytic activity (Figure 5A). Importantly, 2-DG and/or rapamycin treatment resulted in diminished ratio of IL-9⁺ in CD4⁺ T cells and IL-9 expression and secretion and restored the changes in the *Sirt1*^{fllox/fllox}*Cd4-Cre* cells (Figures 5B–5E). Taken together, these data indicate that glycolytic activity is required for SIRT1-directed Th9 cell differentiation.

SIRT1 Deficiency Promotes Th9 Cell Differentiation through an mTOR-HIF1 α -Dependent Glycolytic Pathway

How does SIRT1 regulate glycolytic activity in the course of directing Th9 cell differentiation? To elucidate the mechanism underlying SIRT1 function, we assessed the activation of the IL-4 and/or TGF- β 1 “downstream” pathways, including PU.1 (*Sfpi1*) (Chang et al., 2010), Smad3, STAT6, STAT5, mTOR, and HIF1 α . As expected, the Th9 cell-inducing conditions activated all of these pathways in WT T cells. In contrast, *Sirt1*^{fllox/fllox}

Cd4-Cre T cells exhibited activation of the PU.1, Smad3, STAT6, and STAT5 pathways as the WT cells did, but exhibited both a more potent and sustained activation of phosphorylation of the S6 ribosomal protein and transcription factor HIF1 α (Figures 6A and S6A–S6E). Consistent with this, modest overexpression of SIRT1 in T cells resulted in lower and less sustained inactivation of the phosphorylation of S6 ribosomal protein and HIF1 α (Figure 6B). Thus, SIRT1 is associated with the mTOR-HIF1 α metabolism axis.

To determine whether mTOR or HIF1 α is involved in this regulatory activity, we crossed the *Sirt1*^{fllox/fllox}*Cd4-Cre* and *Mtor*^{fllox/fllox} or *Hif1a*^{fllox/fllox} mice to conditionally delete *Sirt1* and *Mtor* or *Sirt1* and *Hif1a* gene expression in T cells, respectively (called *Sirt1*^{-/-}*Mtor*^{-/-} or *Sirt1*^{-/-}*Hif1a*^{-/-} mice hereafter). The increased IL-9⁺ cells and IL-9 mRNA expression or secretion in *Sirt1*^{fllox/fllox}*Cd4-Cre* cells are significantly restored in *Sirt1*^{-/-}*Mtor*^{-/-} cells or *Sirt1*^{-/-}*Hif1a*^{-/-} cells (Figures 6C–6E and 6F–6H). Consistent with this result, the increased glycolytic activity in *Sirt1*^{fllox/fllox}*Cd4-Cre* cells was also recovered in *Sirt1*^{-/-}*Mtor*^{-/-} cells or *Sirt1*^{-/-}*Hif1a*^{-/-} cells (Figure S6F). To further differentiate the role of mTORC1 and mTORC2, we applied

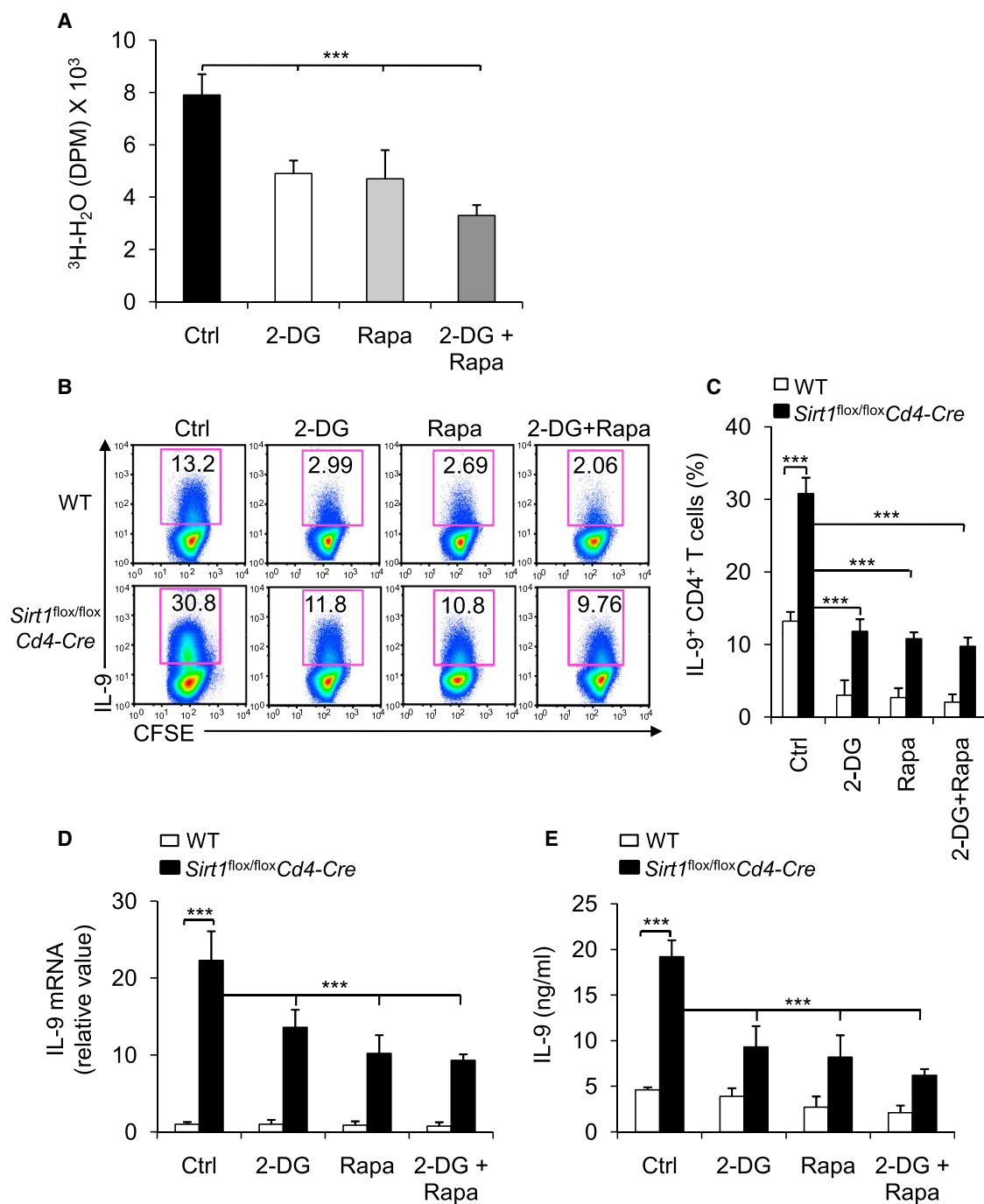


Figure 5. Blocking the Glycolytic Metabolism Pathway Restored SIRT1-Deficiency-Induced Th9 Cell Differentiation

(A) Naive T cells isolated from mouse spleen or peripheral lymph node (PLN) were differentiated under a Th9 cell-inducing conditions for 3 days in the presence of vehicle (Ctrl), 1 mM 2-DG, 50 nM rapamycin (Rapa), or 2-DG+Rapa. Glycolytic activity was measured.

(B and C) Naive T cells were labeled with CFSE and differentiated under Th9 cell-inducing conditions in the presence of vehicle, 2-DG, Rapa, or 2-DG+Rapa, followed by intracellular staining of IL-9. A typical figure is shown (B) and the frequencies of IL-9⁺ cells are summarized (C).

(D) IL-9 expression by the CD4⁺ T cells isolated from naive T cells in WT or *Sirt1*^{flx/flx} *Cd4-Cre* mice differentiated under Th9 cell-inducing conditions and stimulated for 24 hr with anti-CD3 for real-time PCR analyses. Expressions in the WT Ctrl groups were set to 1.

(E) The supernatant was collected and the IL-9 secretion was detected with ELISA

Data are representative of three independent experiments (n = 3–10). ***p < 0.001, compared with the indicated groups.

rapamycin with a dose (50 nM) that blocked the IL-9 production induced by SIRT1 deficiency and inhibited the phosphorylation of S6, indicative of mTORC1 activation. However, the phosphorylation of AKT Ser473, indicative of mTORC2 activation, was largely comparable between control and rapamycin-treated groups (Figures S6G–S6J). These results suggest that the mTORC1 signaling is largely responsible for mediating the IL-9 production induced by SIRT1 deficiency. Furthermore, *Sirt1^{flox/flox}Cd4-Cre*-induced HIF1 α was almost completely blocked in *Sirt1^{-/-}Mtor^{-/-}* cells, but blocking glycolysis with 2-DG did not affect the HIF1 α expression (Figures S7A and S7B). *Sirt1^{flox/flox}Cd4-Cre* and *Sirt1^{-/-}Mtor^{-/-}* or *Sirt1^{-/-}Hif1a^{-/-}* did not affect the proliferation and survival of Th9 cells (Figures S7C–S7E). Therefore, mTOR-HIF1 α -dependent glycolytic modulation is required for SIRT1-dependent Th9 cell differentiation.

HIF1 α Directly Binds to *Il9* in SIRT1-Deficiency-Induced Th9 Cells

We hypothesized that the transcription factor HIF1 α might exert an effect on the regulation of the *Il9* promoter in Th9 cells. To investigate this, we searched the *Il9* promoter for potential binding sites for HIF1 α using the Biobase database. We identified four overlapping putative binding sites for HIF1 α upstream of the transcription start site (TSS) (Figure 6I). To determine the *Il9* promoter occupancies by HIF1 α , binding motifs were used to design ChIP experiments. Primer sets flanking the HIF1 α binding at four sites in the *Il9* promoter were designed to amplify the immunoprecipitated ChIP DNA by real-time PCR (Figure 6I). Naive T cells isolated from WT and *Sirt1^{flox/flox}Cd4-Cre* mice were differentiated under Th9-cell-inducing conditions for 3 days and then analyzed by ChIP PCR. We found that HIF1 α bound to sites II and III in the *Il9* promoter in Th9 cells (Figure 6J). The pattern of HIF1 α binding was very similar to that of histone H3 (trimethylK4) antibodies, which supports the notion that these regions are in transcriptional regulation (Figure 6K).

To analyze the functional relevance of the binding of HIF1 α to its target sequences in the *Il9* locus, we investigated the ability of HIF1 α to regulate the activity of the *Il9* promoter in reporter assays. We used the reporter construct pGL4-*Il9*, which contains luciferase gene under the control of the *Il9* promoter. We found that cotransfection of the pGL4-*Il9* luciferase reporter construct with a plasmid encoding constitutively active HIF1 α (CA-HIF1 α) in 293T cells resulted in a dose-dependent significant increase in *Il9* transcription (Figure 6L). Therefore, these data show that binding HIF1 α to the *Il9* promoter activated *Il9* transcription, which was regulated by SIRT1.

SIRT1 Controls IL-9 Production in Human CD4⁺ T Cells

Next, we sought to apply a pharmacological approach to targets in human T cells for the genetic targeting of SIRT1. We first applied Ex527, a selective inhibitor of SIRT1, and SIRT1720, an activator of SIRT1 in human CD4⁺ T cells during Th9 cell induction. Consistent with the results from genetic targeting of SIRT1, we observed that a SIRT1 inhibitor and activator enhanced and reduced, respectively, the IL-9 mRNA expression and secretion, accompanied by alterations in the glycolytic molecule Glut1 (Figures 7A–7C). The experiment was then extended to include rapamycin (an inhibitor of mTOR), 2-ME (an inhibitor of HIF1 α),

and 2-DG (an inhibitor of the glycolytic pathway) to human T cells under Th9 cell-inducing conditions. Pharmacological inhibition of SIRT1 in human T cells largely recapitulated the observations in the genetic and pharmacological targeting of mouse CD4⁺ T cells in terms of the alterations of human T cell IL-9 expression, secretion, and glycolytic-associated molecules (Figures 7D–7F).

Consistent with the results in mouse cells, SIRT1 siRNA efficiently silenced SIRT1 expression in human CD4⁺ T cells and upregulated the expression of IL-9 and Glut1 (Figures 7G–7I). As expected, rapamycin, 2-ME, and 2-DG treatment largely reversed these effects (Figure 7H). Thus, the data show that SIRT1 mediates an evolutionarily conserved signaling pathway in both mice and humans.

DISCUSSION

T cell subsets differentiate from CD4⁺ T cells that have been exposed to a specialized cytokine environment. Compared with other T cell subsets that have been reported, Th9 cells are less well characterized. Recently, studies have reported factors that control their development, differentiation, and function (Palmer et al., 2015). Here we have shown that SIRT1-dependent signaling coupled to glycolytic activity negatively controlled the production of IL-9 in CD4⁺ T cells in allergic inflammation and anti-tumor immunity. First, TAK1 selectively suppressed SIRT1 during Th9 cell differentiation. Second, overexpression of SIRT1 reduced the amount of IL-9 in CD4⁺ T cells accompanied by lower glycolytic activity. Third, SIRT1 deficiency increased the amount of IL-9 in CD4⁺ T cells along with inducing a higher glycolytic activity. Fourth, SIRT1-mTOR-HIF1 α -dependent glycolytic activity was required for Th9 cell differentiation. Fifth, HIF1 α directly targeted the IL-9 gene promoter and controlled IL-9 production in CD4⁺ T cells.

Th9 cells are defined by their secretion of IL-9 (Kaplan et al., 2015). The production of IL-9 was first described in activated mouse T cells and subsequently cytokines that promote the differentiation of IL-9-producing T cells in culture were identified (Schmitt et al., 1994). Th9 cells have been shown to have roles in various diseases, including allergic inflammation (Cheng et al., 2002) and tumors (Purwar et al., 2012). Mouse studies have provided strong evidence that Th9 cells exert anti-tumor effects, particularly in melanoma (Kim et al., 2015). Here we have demonstrated that SIRT1 deficiency promotes IL-9 production in CD4⁺ T cells and contributes to the responses of allergic airway inflammation and anti-tumor immunity.

SIRT1 is a nuclear NAD⁺-dependent histone deacetylase as well as a key transcriptional regulator of inflammation (Yang et al., 2015). In innate immune cells, SIRT1 has been identified as an important repressor of inflammation (Liu et al., 2015). In T cells, SIRT1 is required to maintain T cell tolerance (Yang et al., 2015). Distinct metabolic programs support the differentiation of CD4⁺ T cells into their separate subsets. However, whether SIRT1 is involved in the metabolism-dependent modulation of intrinsic T cell subset differentiation was not clear. Our data show that TAK1 mediated the selective suppression of SIRT1 in Th9 cells and that the modulation of SIRT1-dependent signaling coupled with glycolytic metabolism is responsible for Th9 cell differentiation and contributed to

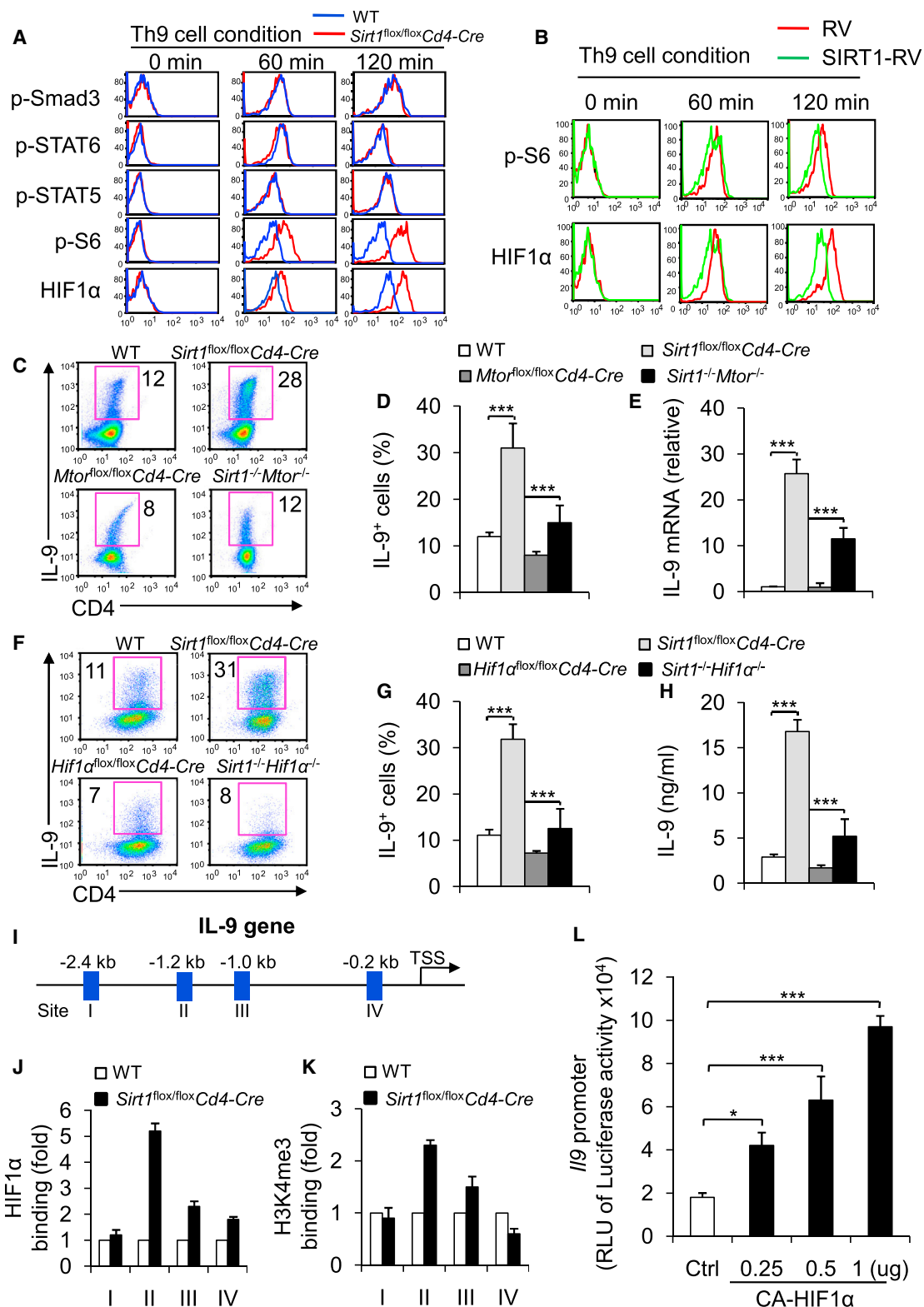


Figure 6. SIRT1 Deficiency Promotes Th9 Cell Differentiation through mTOR-HIF1α-Dependent Signaling Coupled to the Glycolytic Pathway
(A) Activation of the downstream signaling pathways of IL-4 or TGF-β or metabolism. Intracellular staining of the phosphorylation of Smad3, STAT6, and S6 and the expression of HIF1α in the CD4⁺ T cells isolated from WT and *Sirt1*^{flx/flx}Cd4-Cre mice under Th9 cell-inducing conditions.
(B) Intracellular staining of the phosphorylation of S6 and the expression of HIF1α by eGFP⁺ cells sorted from Th9 cell-inducing conditions transduced with RV or SIRT1-RV.

(legend continued on next page)

Th9 cell-associated allergic airway inflammation and anti-tumor immune response.

HIF1 α is a key transcription factor that orchestrates the expression of glycolytic enzymes that can be induced under normoxia as well as hypoxia and plays a central role in pro-inflammatory cytokine production (Cramer et al., 2003). Th17 cells have been shown to rely on glycolysis for their development and maintenance by mTOR-HIF1 α pathway (Shi et al., 2011). HIF1 α deficiency increases the generation of Treg cells, and blocking glycolysis inhibits Th17 cell differentiation (Dang et al., 2011; Shi et al., 2011). Recent studies have shown that SIRT1 directly inactivates HIF1 α through the direct interplay between SIRT1 and HIF1 α in mouse tissues, even in the absence of hypoxia (Lim et al., 2010). SIRT1-HIF1 α axis confers a beneficial effect against certain diseases (Cheng et al., 2014). Consistent with a previous report, although Th9 cell-inducing condition is not optimized for driving Th17 and Treg cell differentiation, HIF1 α upregulation induced by SIRT1 deficiency reciprocally reduced the percentage of Treg cells and increased the percentage of Th17 cells. Importantly, in this condition, SIRT1 deficiency preferentially promoted IL-9 production in CD4⁺ T cells through HIF1 α -dependent glycolysis pathway. Moreover, HIF1 α , downstream of SIRT1-mTOR, directly binds to the *Il9* promoter to promote *Il9* transcription. However, blocking glycolysis did not alter the HIF1 α expression. This suggests that, in addition to HIF1 α 's role in regulating glycolysis, which provides crucial bioenergetics support to drive Th9 cell differentiation, HIF1 α also directly bound to the IL-9 gene promoter to drive IL-9 transcription. Thus, these data suggest that heightened HIF1 α signaling promotes glycolysis and also directly enhances IL-9 expression, both of which are required for SIRT1-directed Th9 cell differentiation.

mTOR is responsible for the glycolytic response upstream of HIF1 α (Cheng et al., 2014). SIRT1 negatively controls mTOR (Liu et al., 2014). mTOR suppression by rapamycin or mTOR deletion promoted the generation of Treg cells (Delgoffe et al., 2009). Th1, Th2, or Th17 cells strongly engage glycolysis via mTOR, whereas Treg cells depend more on the oxidation of lipids (Zeng and Chi, 2015). Consistent with previous studies, we found that SIRT1 deficiency in Th9 cells, but not other T cell subsets, upregulates p-S6 expression, a downstream target of mTOR. Moreover, *Sirt1*^{-/-}*Mtor*^{-/-} cells almost

completely rescue the Th9 cell differentiation and glycolysis activities in *Sirt1*^{fllox/fllox}*Cd4-Cre* cells and also largely block HIF1 α expression. Thus, TAK1-SIRT1-mTOR-HIF1 α signaling coupled with glycolytic pathway is critical for the differentiation of Th9 cells.

Altogether, our work suggested that SIRT1 is selectively suppressed by TAK1 during Th9 cell differentiation and might function as a gatekeeper of downstream mTOR-HIF1 α axis. As such, mTOR-HIF1 α -IL-9 promoter transcriptional regulation coupled with glycolytic activity mechanism modulation is selective for SIRT1-dependent differentiation of Th9 cells that confer protection against tumors and are involved in allergic airway inflammation.

EXPERIMENTAL PROCEDURES

Mice

All animal experiments were performed with the approval of the Animal Ethics Committee of Beijing Normal University, Beijing, China and Fudan University, Shanghai, China. *Sirt1*^{fllox/fllox}, *Mtor*^{fllox/fllox}, *Hif1a*^{fllox/fllox}, and *Cd4-Cre* mice have been described previously (Liu et al., 2009, 2015) and were extensively backcrossed to the C57BL/6 background. WT controls for *Sirt1*^{fllox/fllox}*Cd4-Cre* mice (*Cd4-Cre*; *Sirt1*^{fllox/fllox}) included *Cre*⁺ mice (*Cd4-Cre*; *Sirt1*^{+/+}) to account for the effects of *Cre*. *Rag1*^{-/-} mice (on a C57BL/6 background) were from the Jackson Laboratory. Sex-matched littermates at 7–10 weeks of age were used in the experiments described in this study. All mice were bred and maintained under specific-pathogen-free conditions.

Induction of Allergic Airway Inflammation

Allergic airway inflammation was induced as previously described (Cheng et al., 2002). Mice were sensitized by intraperitoneal injection on days 0 and 14 of OVA (Sigma-Aldrich) adsorbed with aluminum hydroxide (Sigma-Aldrich) at a dose of 10 μ g OVA per 1 mg aluminum hydroxide. From day 21, mice were exposed to aerosolized OVA (1% w/v) for 30 min per day for 5–6 consecutive days. Mice were sacrificed 48 hr after the final intranasal challenge. To evaluate the effects of anti-IL-9 on mouse allergic airway inflammation, mice were given 20 μ g mouse IgG2a (R&D Systems) or anti-IL-9 (R&D Systems) intravenously 30 min before the challenges every day from day 20 to day 24.

Tumor-Bearing Model

Naive T cells were isolated from *Sirt1*^{fllox/fllox}*Cd4-Cre*⁺ and *Sirt1*^{+/+}*Cd4-Cre*⁺ mice, then cultured for 3 days with anti-CD3 and anti-CD28 (both identified above) together with IL-4 and TGF- β 1. These cells were collected and then transferred intravenously into *Rag1*^{-/-} mice (5 $\times$ 10⁶ cells per mouse) on day 0. On the same day, B16 melanoma cells (5 $\times$ 10⁵ cells per mouse) were injected subcutaneously into the side of the abdomen of the mice given

(C and D) Naive T cells were differentiated under Th9 cell-inducing conditions in WT, *Sirt1*^{fllox/fllox}*Cd4-Cre*, *Mtor*^{fllox/fllox}*Cd4-Cre*, or *Sirt1*^{-/-}*Mtor*^{-/-} mice, followed by intracellular staining of IL-9. A typical figure is shown in (C) and the frequencies of IL-9⁺ cells summarized in (D).

(E) IL-9 expression by CD4⁺ T cells isolated from naive T cells in the indicated mice differentiated under Th9 cell-inducing conditions and stimulated for 24 hr with anti-CD3 for real-time PCR analyses. Expressions in WT were set to 1.

(F) Naive T cells were differentiated under Th9 cell-inducing conditions in WT, *Sirt1*^{fllox/fllox}*Cd4-Cre*, *Hif1a*^{fllox/fllox}*Cd4-Cre*, or *Sirt1*^{-/-}*Hif1a*^{-/-} mice, followed by intracellular staining of IL-9. A typical figure is shown in (F) and the frequencies of IL-9⁺ cells are summarized in (G).

(H) IL-9 secretion by CD4⁺ T cells isolated from naive T cells obtained from the indicated mice differentiated under Th9 cell-inducing conditions and stimulated for 24 hr with anti-CD3 for ELISA.

(I) Structural schematics of the four predicated binding sites for HIF1 α in the *Il9* promoter.

(J) ChIP analysis of HIF1 α binding to the *Il9* promoter. Regulation of HIF1 α binding to the four sites within the *Il9* promoter. CD4⁺ T cells from WT and *Sirt1*^{fllox/fllox}*Cd4-Cre* mice were differentiated under Th9 cell-inducing conditions and ChIP-SYBR Green PCR was performed to determine HIF1 α binding to the *Il9* promoter. The Abs are anti-HIF1 α and control IgG. The total input DNA was used for normalization of the data.

(K) ChIP analysis of H3K4 modifications normalized to input controls at the HIF1 α binding four sites within the *Il9* promoter under Th9 cell-inducing conditions for 2 days.

(L) HEK293T cells were transfected with active HIF1 α (CA-HIF1 α) together with a constant amount of an *Il9* promoter-luciferase vector. Cells were lysed 24 hr later, and then the luminescence was measured.

Data are representative of three independent experiments (n = 3–10). *p < 0.05 and ***p < 0.001, compared with the indicated groups. Please see Figures S6 and S7.

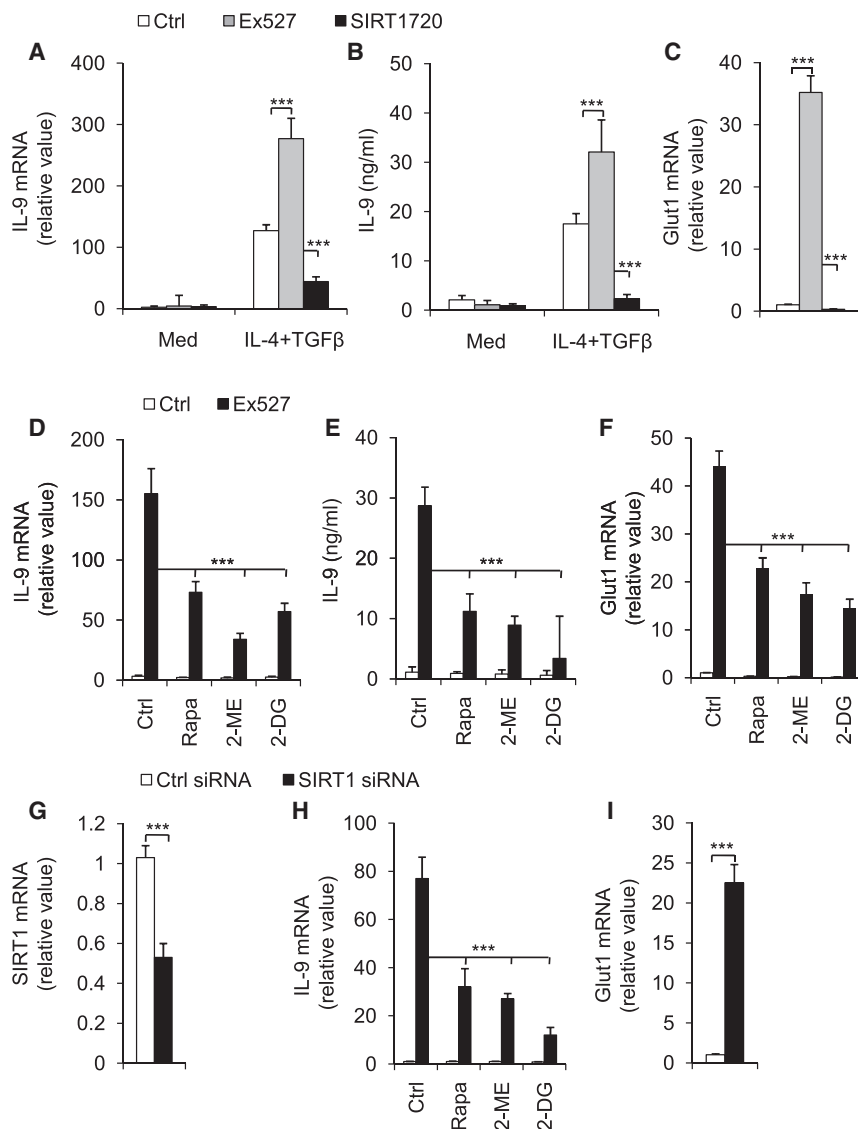


Figure 7. SIRT1 Controls IL-9 Production in Human CD4⁺ T Cells

Naive CD4⁺CD25[−]CD45RA⁺ cells isolated from human peripheral blood mononuclear cells and stimulated for 48 hr with anti-CD3, anti-CD28, and vehicle (medium; med) or with IL-4 and TGF-β1 and the indicated treatment.

(A and D) IL-9 mRNA expression was measured by real-time PCR.

(B and E) IL-9 secretion of supernatant as in (A).

(C and F) Glut1 mRNA expression was analyzed.

(G–I) Expressions of SIRT1 mRNA (G), IL-9 mRNA (H), and Glut1 mRNA (I) in human naive CD4⁺ T cells transfected with control siRNA (Ctrl siRNA) or SIRT1-specific siRNA (SIRT1 siRNA) and stimulated for 48 hr with anti-CD3 and anti-CD28 plus TGF-β1 and IL-4 and the indicated treatment (expressions in Ctrl siRNA were set to 1).

Retroviral Transduction and RNA Interference of SIRT1

SIRT1 was cloned into the MSCV retroviral vector (Clontech Laboratories), as previously described (Liu et al., 2010). A gene-silencing lentiviral construct was generated by subcloning the gene-specific siRNA sequence into a lentiviral siRNA expression plasmid (pMagic4.1; Sbo-bio). The SIRT1 siRNA sequence of 5'-CAGGTTGCAGGA ATCCAAAG-3' was synthesized (Takala) (Liu et al., 2014).

RNA and Protein Analyses

Real-time PCR was performed with probe sets from Applied Biosystems and normalized against the endogenous control gene (Liu et al., 2015). Immunoblot was performed with the following antibodies: p-S6 and SIRT1 (both from Cell Signaling Technology), HIF1α (R&D Systems), and β-actin (Sigma), as described previously (Liu et al., 2014).

ChIP and Luciferase Assay

Chromatin immunoprecipitation (ChIP) was performed according to the protocol described previously (Lim et al., 2010). Luciferase reporter assays were also performed as described previously

(Dang et al., 2011). The mouse *I/9* promoter region (−2410 to +32) was amplified from genomic DNA by PCR with the forward primer (5'-TCGACGCGTGAT GACCTGtgacctgAC-3') and the reverse primer (5'-TCCCcggggcaGAGGC AAGGATGTATGT-3'). The lowercase letters in the primers were Hind III and KpnI sites that were introduced to allow forced cloning into the pGL4 basic luciferase reporter gene vector (Promega).

Glycolysis Flux Assay

Glycolysis in T cells was determined by measuring the detritiation of [3-³H]-glucose (PerkinElmer) (Liu et al., 2014).

Statistical Analyses

All data are presented as the mean ± SD. Student's unpaired t test for comparison of means was used to compare differences between groups. A p value (alpha-value) of less than 0.05 was considered to be statistically significant.

ACCESSION NUMBERS

The microarray data were deposited into the GEO database under accession number GSE79607.

the transfer of T cells. Mice were treated intraperitoneally with anti-IL-9 or control mouse IgG2a (both identical to the above) every 3 days from day 0, as described previously (Nakatsukasa et al., 2015).

Cell Purification and Culture

Lymphocytes were isolated from the spleen and lymph nodes of mice and sorted on a FACSaria II (Becton Dickinson). The sorted naive T cells (CD4⁺TCR⁺CD62L^{hi}CD44^{lo}CD25[−]) were used for in vitro culture as described (Liu et al., 2010). T cells were activated with 2 μg/mL anti-CD3 (2C11; Bio X Cell), 2 μg/mL anti-CD28 (37.51; Bio X Cell), and 100 U/mL human IL-2 (R&D Systems). For Th9 cell differentiation, cultures were supplemented with 10 ng/mL IL-4 (R&D Systems), 2 ng/mL TGF-β1 (R&D Systems), and, for certain experiments only, TGF-β1 and IL-4, as indicated. After 5–6 days culture, differentiated T cells were restimulated for 24–48 hr with plate-bound anti-CD3 (1 μg/mL), and the cell-free supernatants collected after centrifugation were stored at −80°C until use. The amount of IL-9 produced was determined by ELISA (BD Bioscience). T cell proliferation was determined by pulsing the cells with [³H] thymidine for the final 12–16 hr of culture or by labeling with CFSE (carboxyfluorescein diacetate succinimidyl diester; Invitrogen). Flow cytometry was performed with antibodies from eBioscience or BD Biosciences.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.immuni.2016.05.009>.

AUTHOR CONTRIBUTIONS

Y.W. and Y.B. designed and conducted the experiment with cells and mice and analyzed data; X.C. and C.L. did some gene expression analysis; Y. Li, Z.Z., J.W., Y. Lu, Q.Y., H.S., and H.Y. assisted with analysis and interpretation of experiments and results; and G.L. developed the concept, designed and conducted the experiments, analyzed data, wrote the manuscript, and provided overall direction.

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Supplemental Information

Histone Deacetylase SIRT1

Negatively Regulates the Differentiation

of Interleukin-9-Producing CD4⁺ T Cells

Yu Wang, Yujing Bi, Xi Chen, Chunxiao Li, Yan Li, Zhengguo Zhang, Jian Wang, Yun Lu, Qing Yu, Huilin Su, Hui Yang, and Guangwei Liu

Supp. Fig. 1

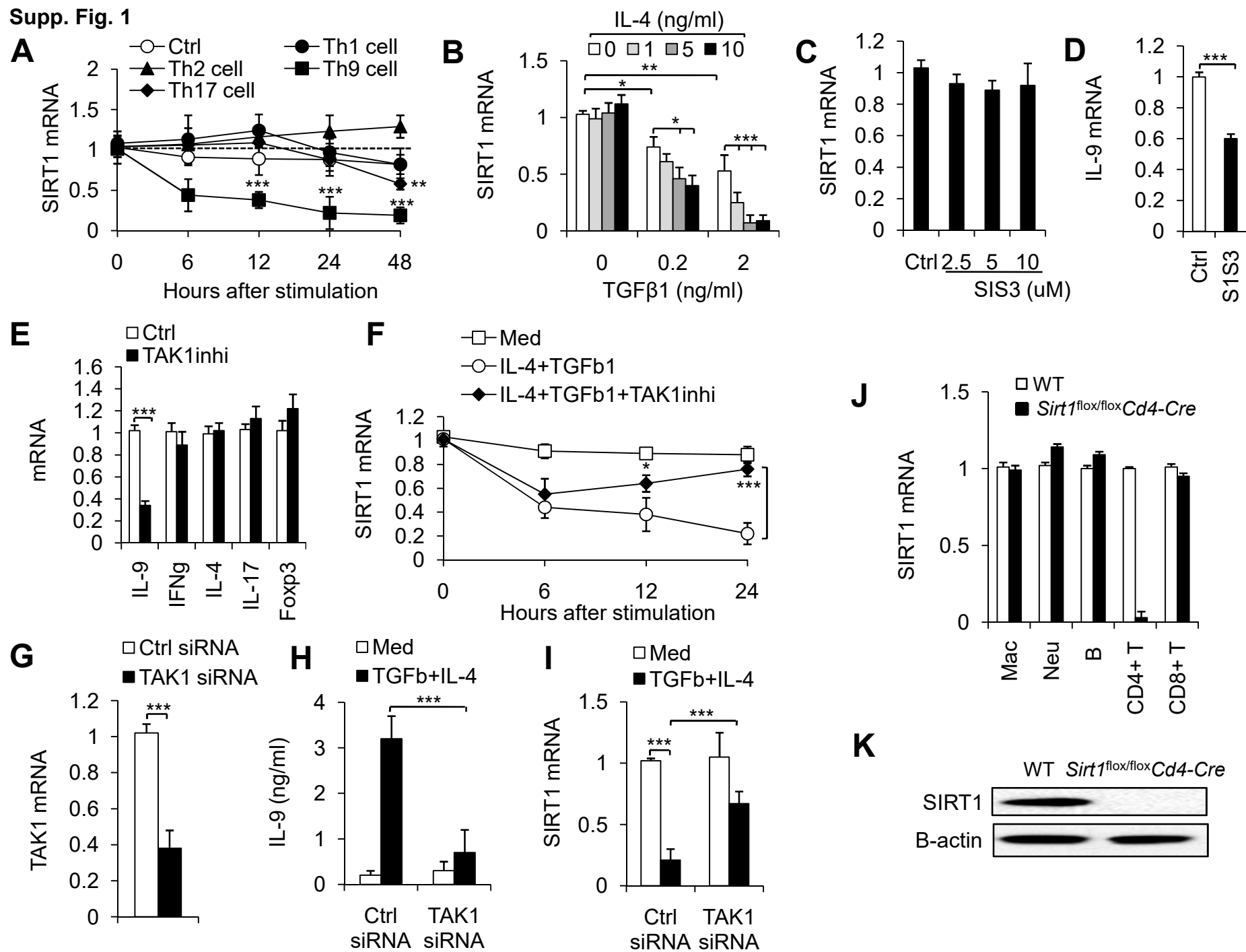


Figure S1. SIRT1 expression in WT or *Sirt1*^{flox/flox}*Cd4-Cre* mice. Related to Fig.1.

(A) SIRT1 mRNA expression as examined by qPCR analyses in T cells activated by various T cell subset-inducing conditions for different time course (Expressions in naïve T cells were set to 1). (B) Naïve T cells were activated by anti-CD3/CD28 in the presence of various concentration of TGFβ1 and/or IL-4 for 24 h and SIRT1 expression was analyzed by qPCR (Expressions in activated without TGFβ1 and IL-4 were set to 1). (C-D) Naïve T cells were differentiated for 3 d under Th9 cell condition in the presence of S1S3 (an inhibitor of Smad3) and SIRT1 (C) and IL-9 mRNA (D) were analyzed (Expressions in Ctrl groups were set to 1). (E) Indicated factors were determined under various T cell subset conditions for 3 d in the presence or absence of TAK1 inhibitor (5z-7-oxozeaenol, sigma, 50 nM). Expressions in Ctrl groups were set to 1. (F) Naïve T cells were activated by indicated condition for different time course and SIRT1 mRNA expression was determined by qPCR. Expressions in naïve T cells were set to 1. (G-I) Expression of TAK1 (G), IL-9 production (H) and SIRT1 mRNA expression (I) in naïve CD4⁺ T cells transfected with control siRNA (Ctrl siRNA), or TAK1-specific siRNA (TAK1 siRNA), and stimulated for 3 d with anti-CD3 and anti-CD28 plus TGFβ1 and IL-4 (Presented in the same manner as in F. Expressions in Ctrl siRNA at med groups were set to 1). (J) Macrophages (CD11b⁺F4/80⁺), Neutrophils (CD11b⁺Ly6G⁺), B cells (CD19⁺), CD4⁺ T cells (CD4⁺ TCR⁺), and CD8⁺ T cells (CD8⁺ TCR⁺) were isolated from WT or *Sirt1*^{flox/flox}*Cd4-Cre* mouse spleen and the purity was confirmed by flow cytometry, and SIRT1 expressions were determined by qPCR (Expressions in WT were set to 1). (K) CD4⁺ T cells isolated from WT and *Sirt1*^{flox/flox}*Cd4-Cre* mice and SIRT1 expressions were determined by immunoblot. Data in Fig. S1 are shown as mean ± SD, n=3-4, from one of three independent experiments. **P*<0.05, ***P*<0.01 and ****P*<0.001, compared with the indicated groups.

Supp. Fig. 2

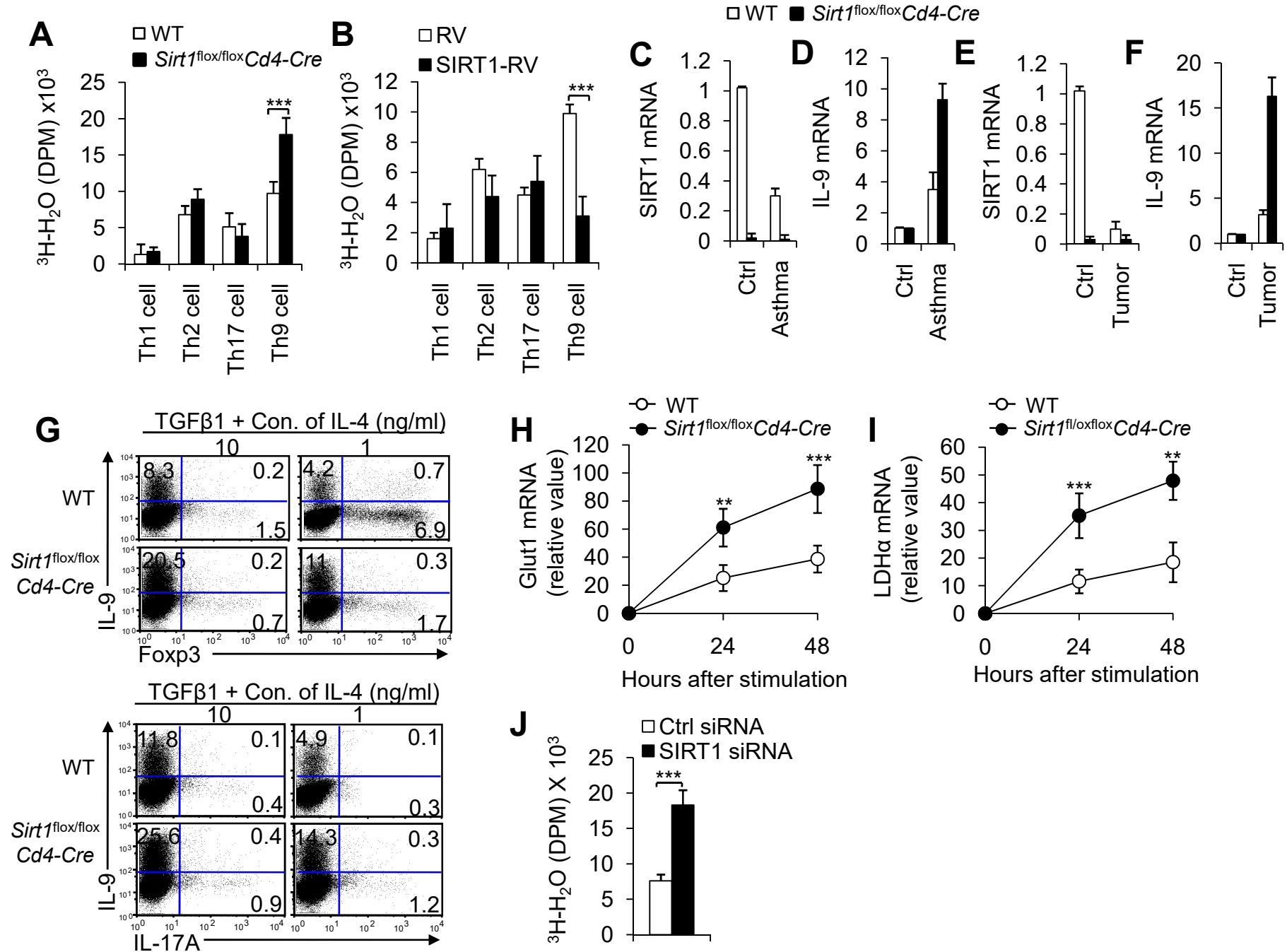


Figure S2. SIRT1 deficient or silencing promotes IL-9 expressions and enhances the glycolytic activity of T cells under Th9 cells-inducing condition. Related to Fig. 1 and 3.

(A) Naïve T cells from WT and *Sirt1*^{flox/flox}*Cd4-Cre* mice were differentiated under various T cell subset-inducing conditions, followed by measured of glycolytic activity. (B) eGFP⁺ cells sorted from various T cell subset-inducing condition transduced with control retrovirus (RV) or *Sirt1*-expressing retrovirus (SIRT1-RV) were measured for glycolytic activity. (C-D) Mice were sensitized intraperitoneal injection on days 0 and 14 of ovalbumin (OVA, Sigma-Aldrich) adsorbed with aluminum hydroxide (Sigma-Aldrich) at a dose of 10 µg OVA per 1 mg aluminum hydroxide. From day 21, mice were exposed to aerosolized OVA (1% w/v) for 30 min per day for 5-6 consecutive days. Mice were killed 48 h after the final intranasal challenge, T cells were isolated from lung draining lymph node (DLN) and SIRT1 mRNA (C) and *Il9* mRNA (D) were measured with qPCR (Expressions of Ctrl WT groups were set to 1). (E-F) *Rag1*^{-/-} mice given intravenous transfer of WT or *Sirt1*^{flox/flox}*Cd4-Cre* naïve CD4⁺ T cells (stimulated via anti-CD3 and anti-CD28, together TGFβ1 and with IL-4 for 3 d) along with simultaneous subcutaneous injection of B16 melanoma cells on day 0. After 30 days, CD4⁺ T cells were isolated from mouse tumor tissue in WT or *Sirt1*^{flox/flox}*Cd4-Cre* mice and SIRT1 mRNA (E) and *Il9* mRNA (F) were measured with qPCR (Expressions of Ctrl WT groups were set to 1). (G) Naïve T cells from WT and *Sirt1*^{flox/flox}*Cd4-Cre* mice were differentiated under Th9 cell-inducing conditions, followed by measured of the expressions of IL-9 and Foxp3 (upper panel) or IL-9 and IL-17 (lower panel) with intracellular staining method. (H-I) Naïve T cells from WT and *Sirt1*^{flox/flox}*Cd4-Cre* mice were differentiated under Th9 cell conditions, followed by measured of mRNA expression of glycolytic molecules and showed the time course changes of glycolytic molecules (Expressions in naïve T cells were set to 1). (J) Naïve CD4⁺ T cells from WT or *Sirt1*^{flox/flox}*Cd4-Cre* mice transfected with control siRNA (Ctrl siRNA) or SIRT1-specific siRNA (SIRT1 siRNA) and stimulated for 48 h with anti-CD3 and anti-CD28 plus TGFβ1 and IL-4, followed by measured of glycolytic activity. Data in Fig. S2 are shown as mean ± SD, n=3-5, from one of three independent experiments. ***P*<0.01 and ****P*<0.001, compared with the indicated groups.

Supp. Fig. 3

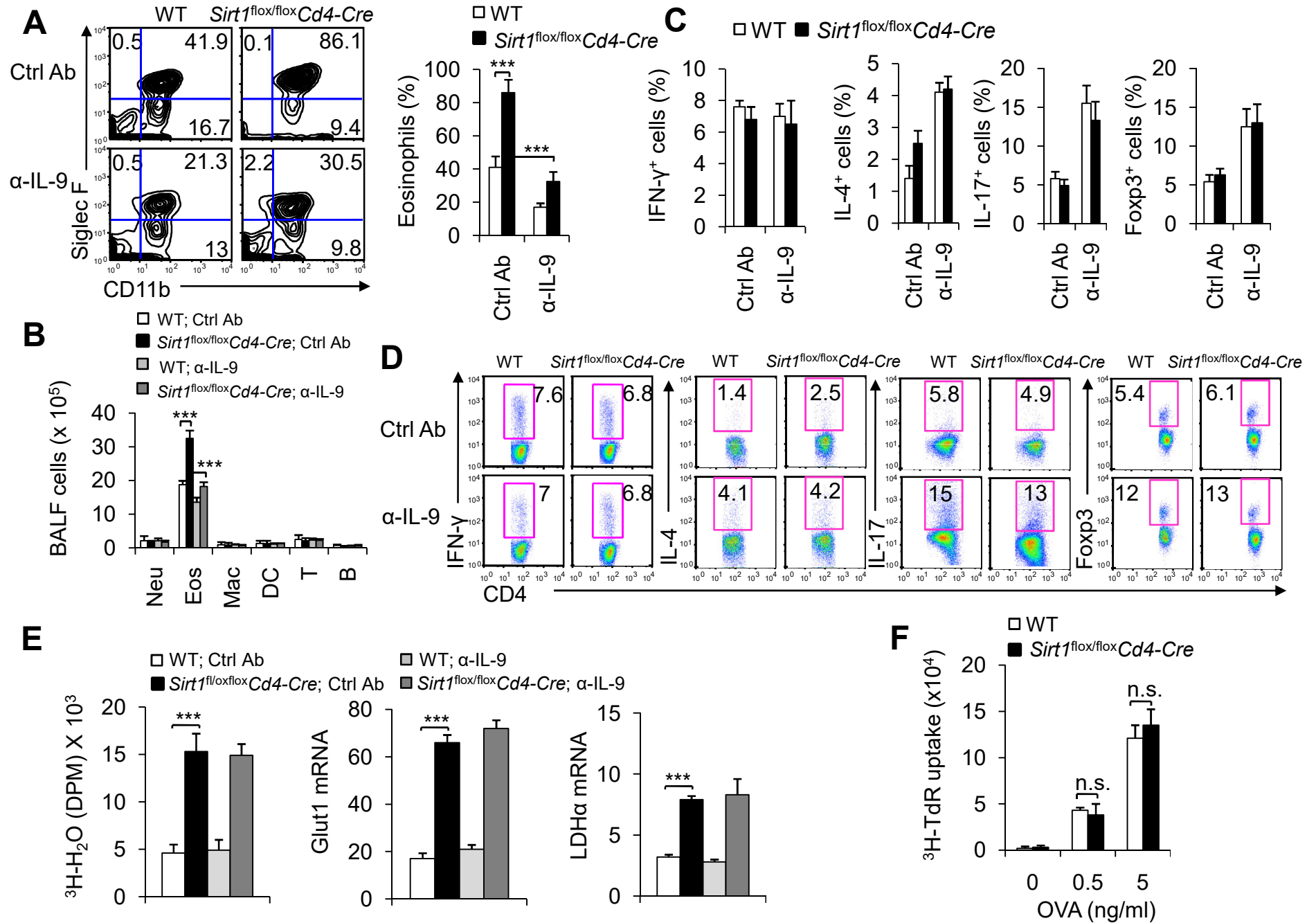


Figure S3. IL-9 produced by SIRT1 deficient CD4⁺ T cells is involved in the development of allergic airway inflammation. Related to Fig. 4.

Mice were sensitized intraperitoneal injection on days 0 and 14 of ovalbumin (OVA, Sigma-Aldrich) adsorbed with aluminum hydroxide (Sigma-Aldrich) at a dose of 10 µg OVA per 1 mg aluminum hydroxide. From day 21, mice were exposed to aerosolized OVA (1% w/v) for 30 min per day for 5-6 consecutive days. Mice were killed 48 h after the final intranasal challenge. **(A-E)** WT and *Sirt1^{flox/flox}Cd4-Cre* mice sensitized and challenged intranasally with OVA on day 0, then treated with anti-IL-9 (α-IL-9) or isotype-matched control antibody (Ctrl Ab) every 3 d from day 0. **(A)** The percentages of eosinophils in BALF fluids of WT and *Sirt1^{flox/flox}Cd4-Cre* were detected by flow cytometry (left), and the frequencies of eosinophils were summarized (right). **(B)** The indicated cell types were determined with flow cytometry and the absolute cell number were summarized. **(C-D)** Intracellular IFN-γ, IL-4, IL-17 and Foxp3 expression of CD4⁺ T cells isolated from lung DLN in WT and *Sirt1^{flox/flox}Cd4-Cre* mice and the frequencies of positive cells were summarized **(C)**, and typical figures were showed **(D)**. **(E)** CD4⁺ T cells isolated from lung DLN, followed by measured of glycolytic activity and mRNA expression of indicated glycolytic molecules. Expressions are presented relative to those of the control gene *Gapdh*. **(F)** WT and *Sirt1^{flox/flox}Cd4-Cre* mice were killed 48 h after the final intranasal challenge, proliferation of DLN cells after *ex vivo* stimulation with OVA for 72 h. Data in Fig. S3 are representative of three independent experiments (n=3-4).

****P*<0.001, compared with the indicated groups. n.s., no significant.

Supp. Fig. 4

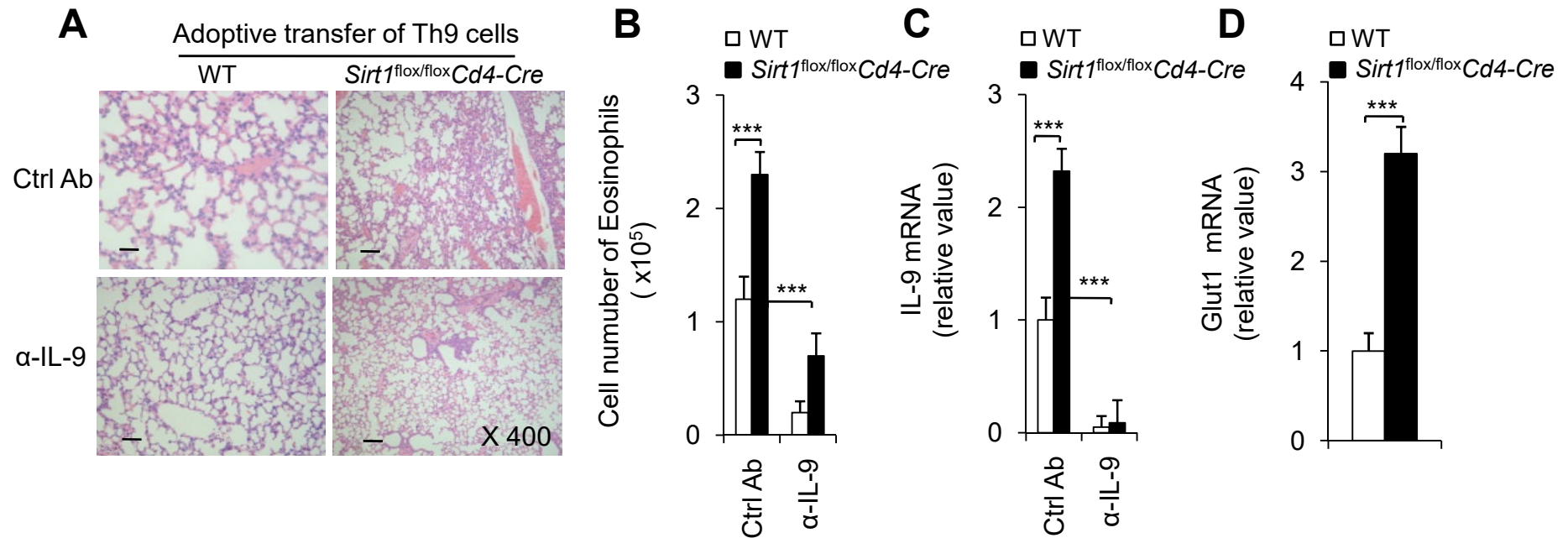


Figure S4. IL-9 produced by SIRT1 deficient CD4⁺ T cells is involved in the development of allergic airway inflammation. Related to Fig. 4.

Rag1^{-/-} mice (n=16) were adoptively transferred with Th9 cells and was sensitized and challenged intranasally with OVA on day 0, then treated with anti-IL-9 (α -IL-9) or isotype-matched control antibody (Ctrl Ab) every 3 d from day 0. **(A)** Hematoxylin and eosin staining of paraffin-embedded sections of lungs from mice challenged as described above 24 h after the final OVA challenge. Original magnification, x 400. **(B)** The absolute total cell number of Eosinophils in BALF. Infiltrating CD4⁺ T cells from lung DLN were isolated and stimulated with anti-CD3 for 24 h, and IL-9 mRNA **(C)** and Glut1 mRNA **(D)** expressions of CD4⁺ T cells were measured by qPCR. Expressions in WT (Ctrl Ab) were set to 1. Data in Fig. S4 are representative of two independent experiments (n=16).

****P*<0.001 compared with the indicated groups.

Supp. Fig. 5

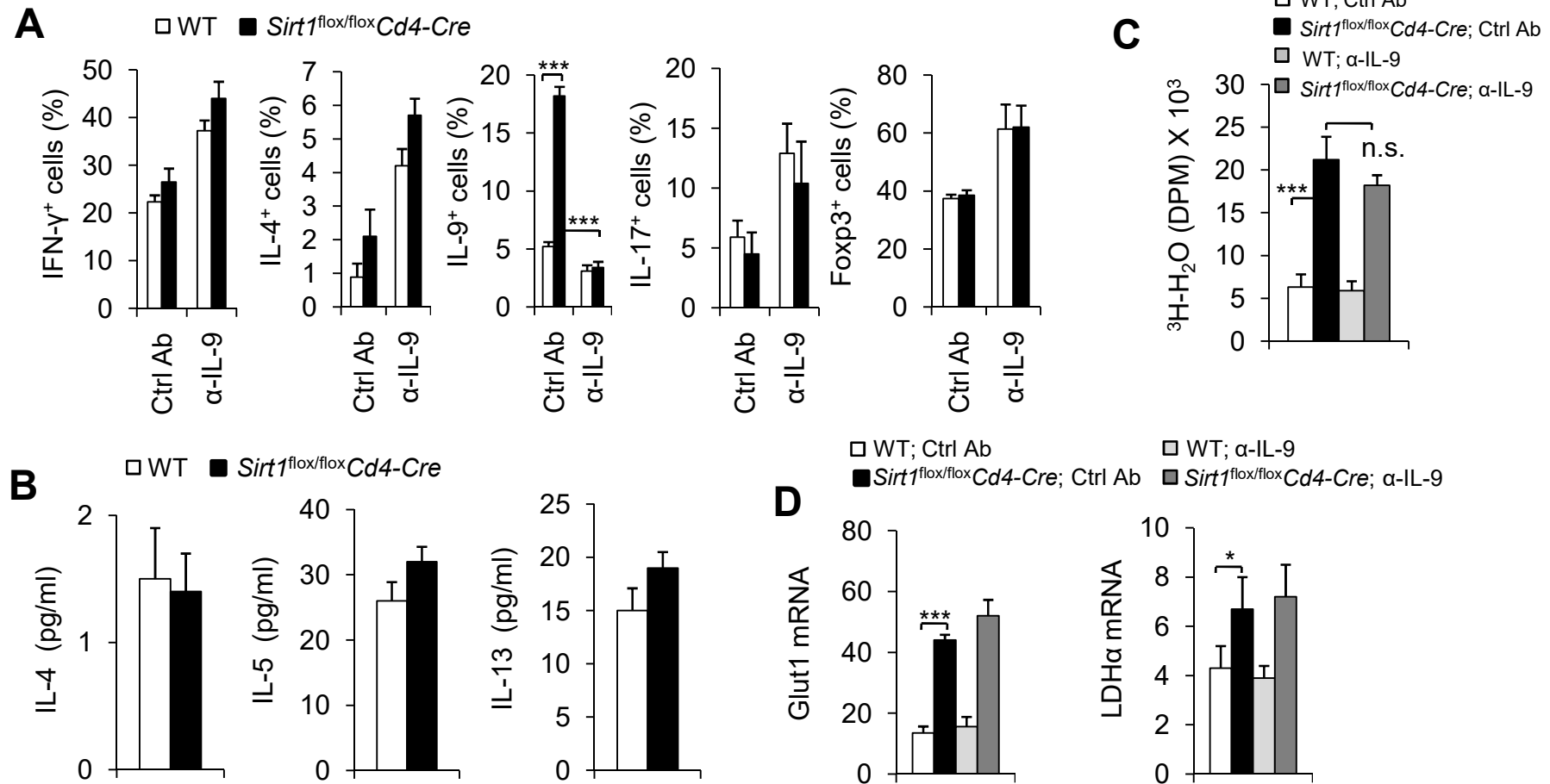


Figure S5. IL-9 produced by SIRT1 deficient CD4⁺ T cells is involved in delaying tumor growth. Related to Fig. 4.

Rag1^{-/-} mice given intravenous transfer of WT or *Sirt1*^{flox/flox} Cd4-Cre naïve CD4⁺ T cells (stimulated via anti-CD3 and anti-CD28, together TGF β 1 and with IL-4 for 3 d) along with simultaneous subcutaneous injection of B16 melanoma cells on day 0, then treated with anti-IL-9 (α -IL-9) or isotype-matched control antibody (Ctrl Ab) every 3 d from day 0. **(A)** Intracellular IFN- γ , IL-4, IL-9, IL-17, and Foxp3 expression of CD4⁺ T cells isolated from tumor infiltrating cells in WT and *Sirt1*^{flox/flox} Cd4-Cre mice and the frequencies of positive cells were summarized. **(B)** CD4⁺ T cells were isolated from solid tumor tissue and were stimulated for 72 h with anti-CD3 and anti-CD28 (identical with above). ELISA of indicated cytokine in supernatant of CD4⁺ T cells. **(C)** CD4⁺ T cells isolated from WT and *Sirt1*^{flox/flox} Cd4-Cre mouse tumor, followed by measured of glycolytic activity. **(D)** CD4⁺ T cells were isolated from solid tumor tissue and followed by measured of mRNA expression of indicated glycolytic molecules. Data in Fig. S5 are representative of three independent experiments (n=3-5). * P <0.05, and *** P <0.001, compared with the indicated groups. n.s., no significant.

Supp. Fig. 6

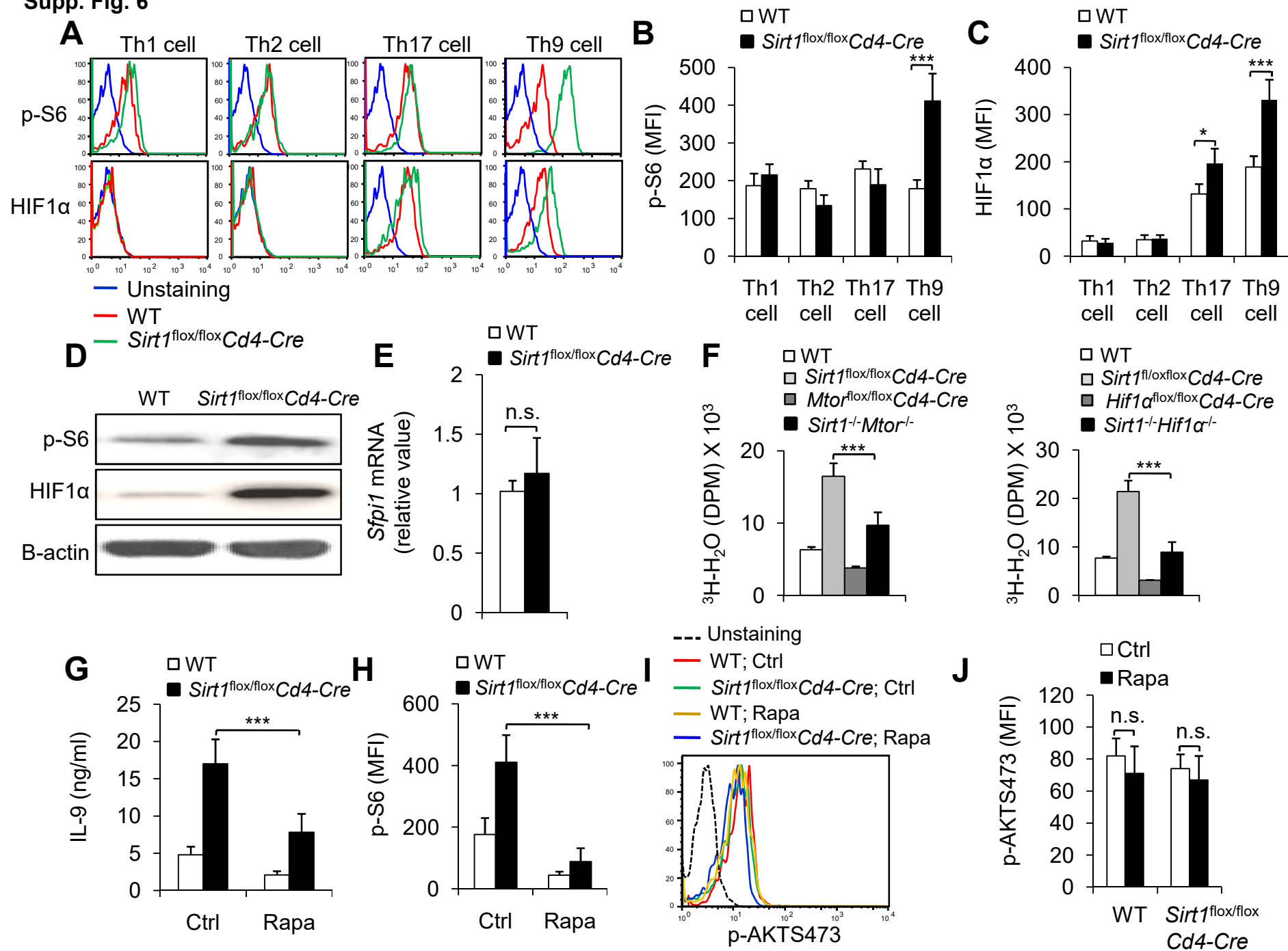


Figure S6. mTOR and HIF1 α -dependent glycolysis is upregulated by SIRT1 deficiency CD4⁺ T cells under Th9 cell-inducing condition. Related to Fig.6.

(A-C) p-S6 and HIF1 α expression of various T cell subset. Intracellular staining of phosphorylation of S6, and expression of HIF1 α in the CD4⁺ T cells isolated from WT and *Sirt1*^{flox/flox}*Cd4-Cre* mice under various T cell subset-inducing condition. Typical figure showed in A, and the MFI of p-S6 (B) and HIF1 α (C) of T cells summarized. (D) Immunoblot of phosphorylation of S6 and expression of HIF1 α in the CD4⁺ T cells isolated from WT and *Sirt1*^{flox/flox}*Cd4-Cre* mice under Th9 cell-inducing condition. (E) The mRNA expression of *Sfp1* (gene encoding PU.1) by WT or *Sirt1*^{flox/flox}*Cd4-Cre* mouse CD4⁺ T cells under Th9 cell-inducing condition and stimulated for 24 h with anti-CD3. Expressions in WT groups were set to 1. (F) Naïve T cells were differentiated under Th9 cell conditions in WT, *Sirt1*^{flox/flox}*Cd4-Cre*, *Mtor*^{flox/flox}*Cd4-Cre* or *Sirt1*^{-/-}*Mtor*^{-/-} mice (left panel) or in WT, *Sirt1*^{flox/flox}*Cd4-Cre*, *Hif1* α ^{flox/flox}*Cd4-Cre* or *Sirt1*^{-/-}*Hif1* α ^{-/-} mice (right panel) followed by measured of glycolytic activity. (G-J) Naïve CD4⁺ T cells isolated from WT or *Sirt1*^{flox/flox}*Cd4-Cre* mice were differentiated under Th9 cell-inducing conditions for 3 d in the presence of vehicle (Ctrl), 50 nM rapamycin (Rapa). The CD4⁺ T cells were stimulated for 24 h with anti-CD3, supernatant was collected, and IL-9 secretion was determined with ELISA (G). Intracellular staining of the phosphorylation of S6 (H) and AKT Ser473 (I-J) in the CD4⁺ T cells. The typical figure of p-AKT Ser473 showed in I. The MFI of p-S6 or p-AKT Ser473 of T cells summarized (H or J). Data in Fig. S6 are shown as mean $\pm$ SD, n=3, from one of two independent experiments. **P*<0.05, ****P*<0.001, compared with the indicated groups. n.s., no significant.

Supp. Fig. 7

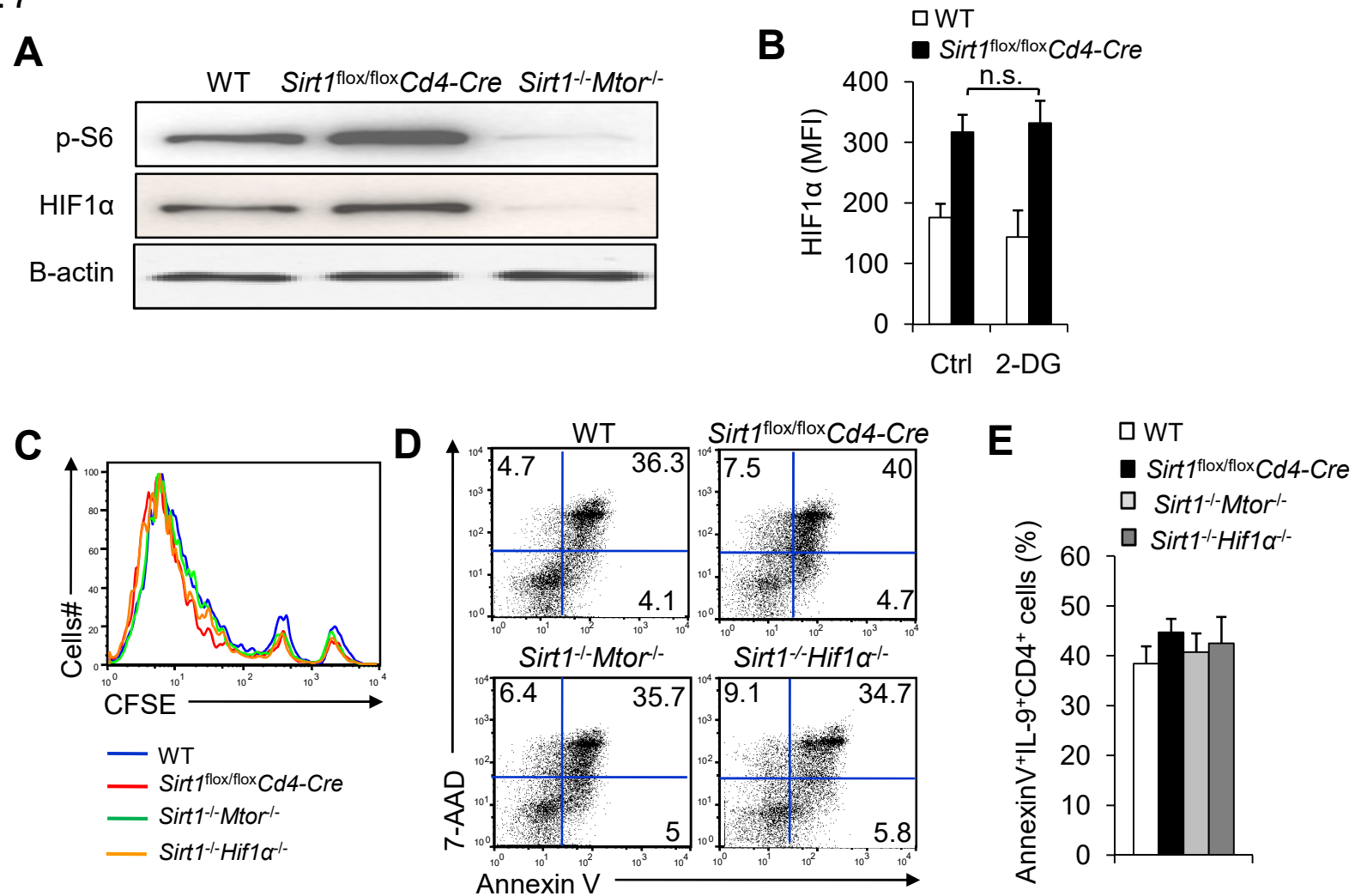


Figure S7. Th9 cell proliferation and survival. Related to Fig. 6.

(A) Immunoblot of p-S6 and HIF1α in Th9 cells. (B) Naïve T cells were differentiated under Th9 cell-inducing condition for 3 d in the presence of vehicle (Ctrl), 1 mM 2-DG. Expression of HIF1α in CD4⁺ T cells was measured and the MFI was summarized. (C-E) Naïve CD4⁺ T cells were isolated from indicated mice and were differentiated under Th9 cell conditions for 3 d. (C) Naïve CD4⁺ T cells were labeled with CFSE and CD4⁺ IL-9⁺ T cell proliferation was determined by CFSE dilution. (D-E) The frequency of apoptotic and dead CD4⁺ IL-9⁺ cells was assessed with Annexin V and 7-AAD staining. Typical figure showed in D and data summarized in E. Data in Fig. S7 are shown as mean ± SD, n=3, from one of three independent experiments. n.s., no significant.

Supplemental Experimental Procedures

Cell Purification and Culture

Lymphocytes were isolated from the spleen and lymph nodes of mice and sorted on a FACS Aria II (Becton Dickinson). The sorted naïve T cells ($CD4^{+}TCR^{+}CD62L^{high}CD44^{low}CD25^{-}$) were used for *in vitro* culture as described (Liu et al., 2010). T cells were activated with 2 ug/ml anti-CD3 (2C11; Bio X Cell), 2 ug/ml anti-CD28 (37.51; Bio X Cell) and 100 U/ml human IL-2 (R&D system). For Treg cell differentiation, cultures were supplemented with 2 ng/ml human TGF β 1 (R&D system). For Th1 cell differentiation, cultures were supplemented with 3.5 ng/ml IL-12 (R&D system) and 10 ug/ml anti-IL-4 (11B11; Bio X Cell). For Th2 cell differentiation, cultures were supplemented with 10 ng/ml IL-4 and 10 ug/ml anti-IFN- γ (all from R&D system). For Th17 cell differentiation, cultures were supplemented with 10 ug/ml anti-IL-2, 10 ug/ml IL-23, 2 ng/ml TGF β 1 and 10 ng/ml IL-6 (all from R&D system).

Flow cytometry

For FCM analysis of cell surface markers, cells were stained with antibodies in PBS containing 0.1% (wt/vol) BSA and 0.1% NaN₃ as described previously (Liu et al., 2009). The following antibodies were obtained from eBioscience: anti-CD4 (GK1.5) and anti-CD11b (M1/70). Anti-CD45 (TU116), anti-Siglec F (E50-2440), anti-F4/80 (BM8), anti-CD19 (1D3) and anti-CD11c (N418) antibodies were obtained from BD Biosciences. Anti-Histone H3 (tri methyl K4; ab8580) were obtained from Abcam. Anti-HIF1 α (IC1935P) was obtained from R&D system.

Intracellular cytokine IFN- γ (XMG1.2; BD Biosciences), IL-4 (11B11; BD Biosciences), IL-9 (D9302C12; BD Bioscience), IL-17A (TC11-18H10; BD Biosciences) staining, T cells were stimulated for 5 h with phorbol 12-myristate 13-acetate and ionomycin in the presence of monensin before being stained according to the manufacturer's instructions (BD Biosciences). Foxp3 (FJK-16s; eBioscience) and HIF1 α (IC1935P; R&D system) staining were done according to the manufacturer's instructions. For phosphorylated signaling protein staining, activated cells with IL-4 (10 ng/ml), TGF β 1 (2 ng/ml), anti-CD3 (145-2c11; BD Biosciences) and anti-CD28 (37.51; BD Biosciences), were immediately fixed with Phosflow Perm buffer (BD Biosciences), were made permeable with Phosflow Lyse/Fix buffer (BD Biosciences) and were stained with anti-p-Smad3 (C25A9; Cell Signaling Technology), anti-p-STAT6 (9361; Cell Signaling Technology), anti-p-STAT5 (C71E5; Cell Signaling Technology), anti-p-AKT (Ser 473; D9E; Cell Signaling Technology), anti-p-S6 (D57.2.2E; Cell Signaling Technology). Flow cytometry data were acquired on a FACSCalibur (Becton Dickinson, San Diego, CA) or an Epics XL bench-top flow cytometer (Beckman Coulter, CA), and data were analyzed with FlowJo

(TreeStar, San Carlos, CA). Cell numbers of various populations were calculated by multiplication of the total cell number by the percentages of each individual population from the same mouse, followed by averaging.

Retroviral transduction

SIRT1 were cloned into the MSCV retroviral vector (Clontech Laboratories, Mountain View, CA), as previously described (Liu et al., 2014). Phoenix-Eco packaging cells (ABP-RVC-10001; Allele Biotechnology, San Diego, CA) were transfected with Lipofectamine (Invitrogen), and recombinant retrovirus was collected 48 h after transfection. After 2 d of differentiation, T cells were transduced with retroviral supernatant by spin inoculation (650 x g for 1 h), as described (Liu et al., 2010). Cells were sorted based on the expression of GFP by flow cytometry and performed the cytokine production and gene-expression analysis.

Quantitative RT-PCR

Total RNA of T cells was extracted with the RNeasy kit (Qiagen, Dusseldorf, Germany), and cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). An ABI 7900 real-time PCR system was used for quantitative PCR, with primer and probe sets obtained from Applied Biosystems (Carlsbad, CA, USA). Results were analyzed using SDS 2.1 software. The expression of each target gene is presented as the 'fold change' relative to that of control samples (2-DDCT), as described previously (Liu et al., 2014).

Chromatin immunoprecipitation assay

Naïve CD4⁺ T cells isolated from spleen and/or lymph node were cultured under Th9 cell-inducing condition. Chromatin immunoprecipitation (ChIP) was performed according to the protocol described previously (Lee et al., 2015; Lim et al., 2010). Structural schematics of four predicated binding sites for HIF1 α in the *I/I9* promoter. After culture 24 h, cells were collected and then were fixed for 10 min at room temperature with 1% formaldehyde and lysed in lysis buffer (Diagenode). Lysates were sonicated, followed by precipitation with anti-HIF1 α antibodies or anti-Histone H3 antibodies for overnight. Immunoprecipitated complex were reverse-cross linked and DNA was eluted. Quantitative PCR was performed and analyzed.

Luciferase assay

Luciferase reporter assays were done as described previously (Liao et al., 2014). The mouse *I/I9* promoter region (-2410 to +32) was amplified from genomic DNA by PCR with forward primer and reverse primer. Lowercase letters in primers were Hind III and Kpn I sites was introduced to allow forced cloning into the pGL4 basic luciferase reporter gene vector (Promega). HEK293T cells were transfected with the reporter vector together with expression plasmids containing cDNA encoding mouse HIF1 α (CA-HIF1 α) and

the pRL-TK vector (Dual luciferase reporter assay system; Promega) as an internal control to exclude differences in transfection efficiency through the use of Lipofectamine 2000 method (Invitrogen). Cells were cultured for 24 h before harvesting, *IL9* promoter activity was assessed by a Dual-Luciferase reporter assay system according to the manufacturer's instructions (Promega) using a luminometer (TD20/20; Turner Designs).

SIRT1 silenced by RNA interference

A gene-silencing lentiviral construct was generated by subcloning gene-specific siRNA sequence into lentiviral small interfering RNA (siRNA) expression plasmid (pMagic4.1; Sbo-bio, Shanghai, China). SIRT1 siRNA sequence is 5'-CAGGTTGCAGGAATCCAAAG-3' was synthesized (Takala Co. Ltd., Dalian, China), as described previously (Liu et al., 2014). Lentiviruses were harvested from culture supernatant of 293T cells (CL1032; Abgent, San Diego, CA) transfected with siRNA vector. Sorted naïve T cells from mice or human were stimulated for 24 h with anti-CD3 and anti-CD28 (both identical above) and were infected with recombinant lentivirus. After overnight culture, cells were restimulated for 24 h with TGFβ1 and IL-4, followed by real-time PCR analysis of the target mRNA or IL-9 mRNA.

Gene expression profiling by microarray analysis

RNA samples collected from biological triplicates were analyzed using the agilent feature extraction software (version 11.0.1.1) and expression signals were summarized using GeneSpring GX v12.1 software package (Agilent Technologies). Differentially expressed genes were identified through Fold Change filtering. Hierarchical Clustering was performed using the R scripts. The entire microarray data was deposited into the GEO series database under accession number GSE79607.

Glycolysis flux assay

Glycolysis of T cells were determined by measuring the detritiation of [3-³H]-glucose (PerkinElmer) and, 2 h later, the medium was transferred to microcentrifuge tubes containing 50 ul 5N HCL. The microcentrifuge tubes were then placed in 20 ml scintillation vials containing 0.5 ml water, and the virals were capped and sealed. ³H₂O was separated from unmetabolized [3-³H]-glucose by evaporation diffusion for 24 h at room temperature, as described previously (Liu et al., 2014).

Human T cells *in vitro*

Human peripheral blood CD4⁺ T cells (6902K-50a; Cell applications) were purified with a naïve T cell isolation kit (Miltenyi Biotec). Naïve T cells were cultured for 48 h with plate-bound anti-human CD3 (1 ug/ml) and soluble anti-human CD28 (1 ug/ml) with TGFβ1 (2 ng/ml) plus IL-4 (10 ng/ml). Optimized dose of Ex527 (10 μM/L), SIRT1720 (10 μM/L), Rapamycin (50 nM/L), 2-ME (2 μM/L) and 2-DG (1 mmol/L) were used in human T cell culture.

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