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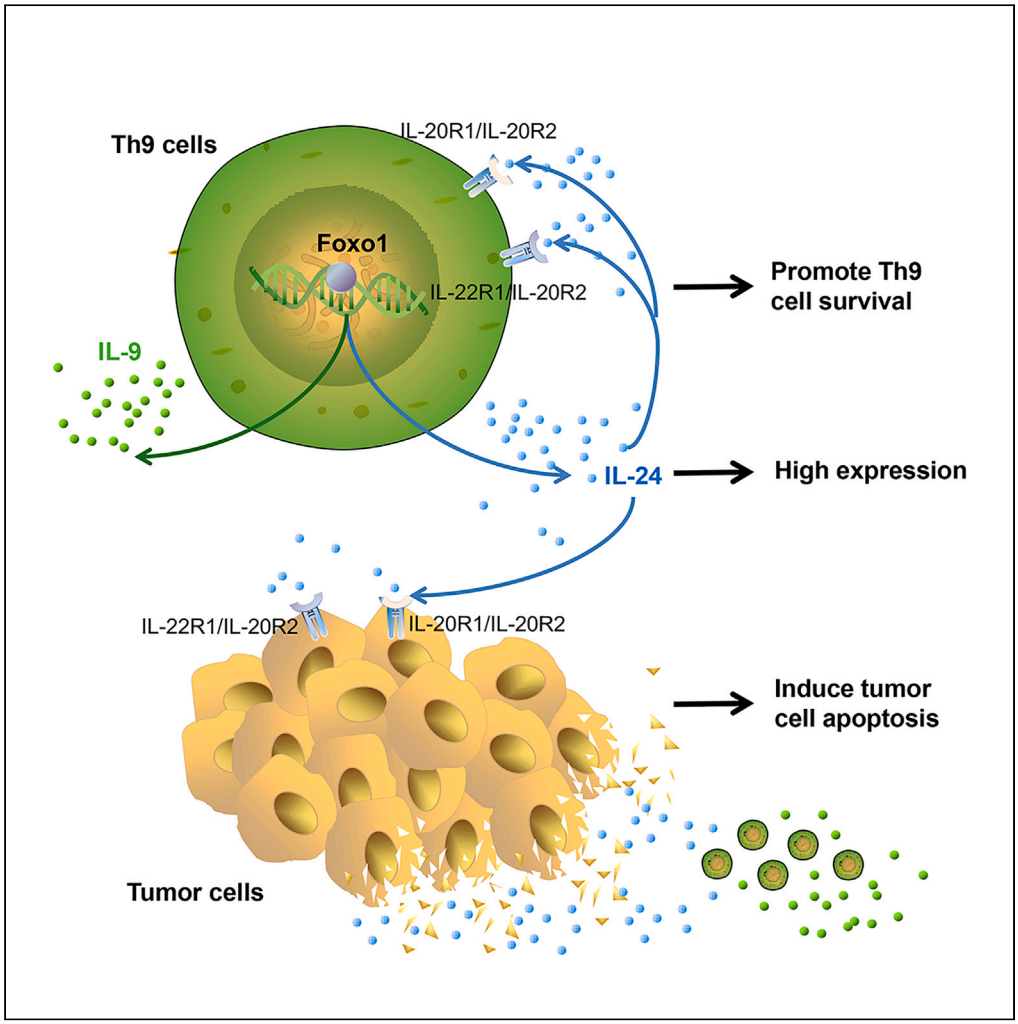

Jintong Chen, Yunwei Zhang, Hua Zhang, ..., Tianxue Qin, Sujun Gao, Siqing Wang

sjgao@jlu.edu.cn (S.G.)
wkerger@jlu.edu.cn (S.W.)

Highlights

Th9 cells express high levels of IL-24

Th9-derived IL-24 contributes to Th9 cell survival

Th9-derived IL-24 exerts the direct antitumor efficacy

Foxo1 contributes to IL-24 expression in Th9 cells

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IL-24 is the key effector of Th9 cell-mediated tumor immunotherapy

Jintong Chen,[1] Yunwei Zhang,[2] Hua Zhang,[3] Mingyue Zhang,[4] He Dong,[4] Tianxue Qin,[2] Sujun Gao,[2,*] and Siqing Wang[1,5,*]

SUMMARY

Th9 cells are powerful effector T cells for cancer immunotherapy. However, the underlying antitumor mechanism of Th9 cells still needs to be further elucidated. Here, we show that Th9 cells express high levels of not only IL-9, but also IL-24. We found that knockout of *Il24* gene in Th9 cells promotes Th9 cell proliferation *in vitro*, but decreases Th9 cell survival *in vitro* and *in vivo*. Interestingly, knockout of *Il24* gene in Th9 cells decreases the tumor-specific cytotoxicity of Th9 cells *in vitro*. In addition, immunotherapy with *Il24* knockout Th9 cells exhibit less tumor inhibition than regular Th9 cells in mouse tumor models. We found that inhibition of Foxo1 by a specific inhibitor downregulates IL-24 expression in Th9 cells and decreases Th9 cell antitumor efficacy *in vivo*. Our results identify IL-24 as a powerful antitumor effector of Th9 cells and provide a target in Th9 cell-mediated tumor therapy.

INTRODUCTION

Th9 cell is a novel $CD4^+$ T cell subset characterized by high expression of interleukin (IL)-9.[1,2] Multiple transcription factors, such as PU.1, interferon regulatory factor 4 (IRF4) and forkhead box O 1 (Foxo1), are shown to be involved in Th9 cell differentiation and IL-9 expression.[3–5] Th9 cells are originally shown to induce potent antitumor efficacy in a mouse tumor model of melanoma.[6,7] Th9 cells also exhibit potent antitumor properties in a variety of other tumors, including colon cancer,[8,9] and breast cancer,[10] lung adenocarcinoma.[11] We found that dectin-1-activated dendritic cells (DCs) and their derived cytokines can promote Th9 cell differentiation and enhance their antitumor efficacy.[12–14] Based on these observations, Th9 cells may represent promising effector cells for tumor therapy.

The mechanism underlying the antitumor effects of Th9 cells still remains unclear. Th9 cells express a large amount of IL-9. IL-9 is identified as a T cell growth factor[15] and is shown to promote T cell proliferation and survival.[15–17] Th9 cells possess an effector profile with less exhaustion and longer longevity, which may contribute to the antitumor efficacy of Th9 cells.[18] Th9/IL-9 promotes tumor-specific CTL responses.[7] Th9 cell-derived IL-3 prolongs the survival of DCs which contribute to the induction of tumor-specific CTLs.[19] Th9 cells secrete granzyme (Gzm) A and GzmB, which may mediate the tumor cytotoxicity of Th9 cells.[6,18] Based on these observations, the mechanism of Th9 cell-mediated antitumor activity needs to be further exploited.

IL-24 is a member of IL-10 gene family.[20] C/EBP-β and c-Jun are the specific transcription factors of IL-24.[21] IL-24 has two heterodimeric receptors, IL-20R1/IL-20R2 and IL-22R1/IL-20R2.[22] IL-24 has two molecular forms: an intracellular form and a secreted form.[23] Intracellular IL-24 plays a tumor suppressive role in various types of tumors by inducing cell apoptosis and autophagy, inhibiting angiogenesis and tumor invasion, and sensitizing tumor cells to radiation therapy and chemotherapy.[24–28] Secreted IL-24 significantly inhibits the proliferation of tumor cells and induced apoptosis of tumor cells.[29,30] IL-24 can promote the secretion of IFN-γ from T cells, and enhance the antitumor cytotoxicity of tumor-specific CTLs.[31] Previous studies show that Th9 cells also express IL-24.[5,32–34] However, the role of IL-24 in Th9 cell-mediated antitumor activity remains unclear.

In this study, we found that IL-24 is highly expressed in Th9 cells. Knockout of *Il24* gene in Th9 cells promotes Th9 cell proliferation *in vitro*, but decreases Th9 cell survival *in vitro* and *in vivo*. Interestingly, knockout of *Il24* gene in Th9 cells decreases the tumor-specific cytotoxicity of Th9 cells *in vitro*. In addition,

[1]Department of Cancer Immunology, First Hospital of Jilin University, Changchun 130061, China

[2]Department of Hematology, First Hospital of Jilin University, Changchun 130061, China

[3]Department of Gastrointestinal Surgery, First Hospital of Jilin University, Changchun 130021, China

[4]Department of Gynecological Oncology, First Hospital of Jilin University, Changchun 130021, China

[5]Lead contact

*Correspondence: sjgao@jlu.edu.cn (S.G.), wkerger@jlu.edu.cn (S.W.)

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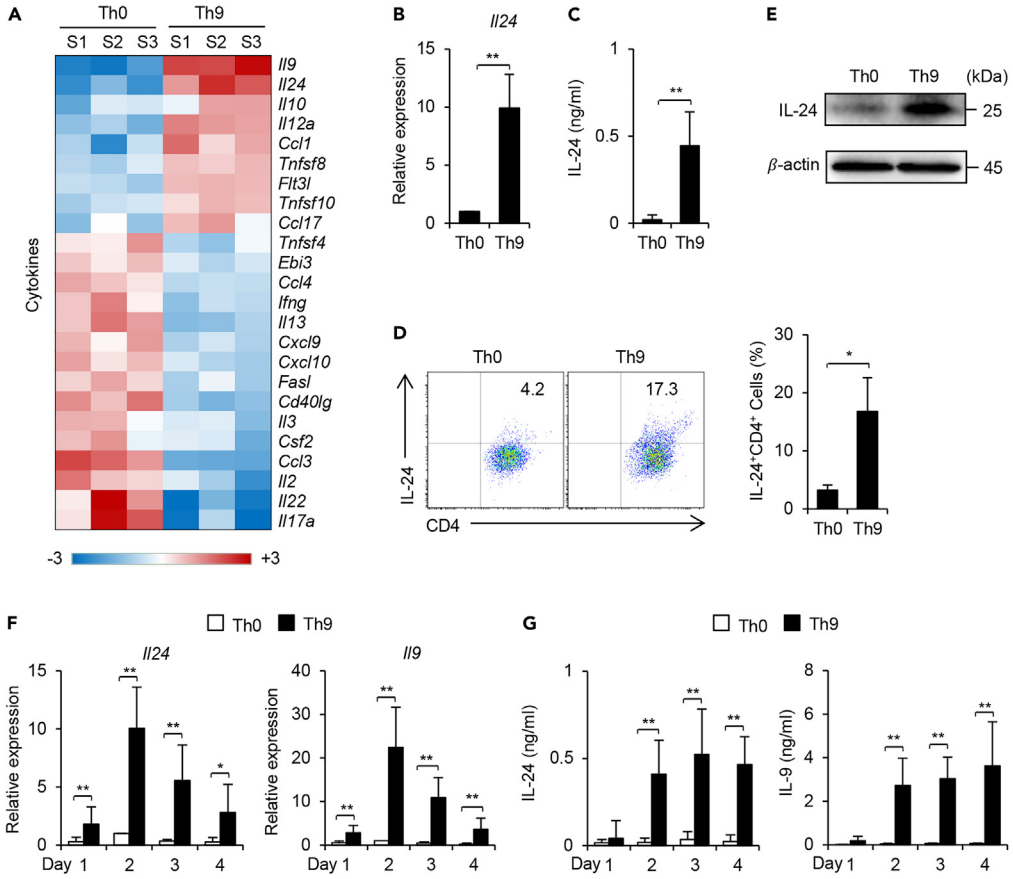

Figure 1. IL-24 is highly expressed in Th9 cells

(A–E) Naive CD4$^+$ T cells isolated from spleens of mice were cultured under Th0- or Th9-polarizing conditions for 2 days.

(A) Cell samples (S1-3) were collected and analyzed by RNA-seq. S1-3: Samples from three independent experiments. The heatmap shows the $\log_2$-fold change of the significantly differentially expressed genes of cytokines and chemokines.

(B) The expression of *Il24* in cultured T cells was assessed by qPCR.

(C) IL-24 secretion in the cultures was assessed by ELISA.

(D) Intracellular IL-24 expression was analyzed by Flow cytometry. Numbers in the dot plots represent the percentages of IL-24$^+$CD4$^+$ T cells. Right, summarized results of three independent experiments obtained as at left.

(E) Western-blots examined IL-24 and β-actin in T cells.

(F and G) Naive CD4$^+$ T cells isolated from spleens of mice were cultured under Th0- or Th9-polarizing conditions. Cells samples were collected at the specified time points.

(F) The expression of *Il24* in cultured T cells was assessed by qPCR.

(G) The secretion of IL-24 and IL-9 in the cultures was analyzed by ELISA. Data are representative of three (D, E) independent experiments or presented as mean ± SD of three (B-D, F-G) independent experiments. *$p < 0.05$; **$p < 0.01$. See also Figures S1–S3.

immunotherapy with *Il24* knockout Th9 cells had less effect than with regular Th9 cells in mouse tumor model. Our results identify IL-24 as a powerful antitumor effector of Th9 cells and provide a target in Th9 cell-mediated tumor therapy.

RESULTS

IL-24 is highly expressed in Th9 cells

We first detected the gene expression spectrum of Th9 cells. Among the total of 26,423 mouse genes, we identified 293 genes which are differentially expressed in Th9 cells compared to Th0 cells (Table S1). Among these 293 genes, 24 genes of cytokines and chemokines are differentially expressed, including 9 up-regulated genes and 15 down-regulated genes (Figure 1A). Besides *Il9* which is highly expressed in Th9 cells, we found that *Il24* was the top 2 up-regulated gene of cytokines and chemokines in Th9 cells (Figure 1A). The up-regulation of IL-24 by Th9 cells was confirmed by qPCR, ELISA, Flowcytometry and western

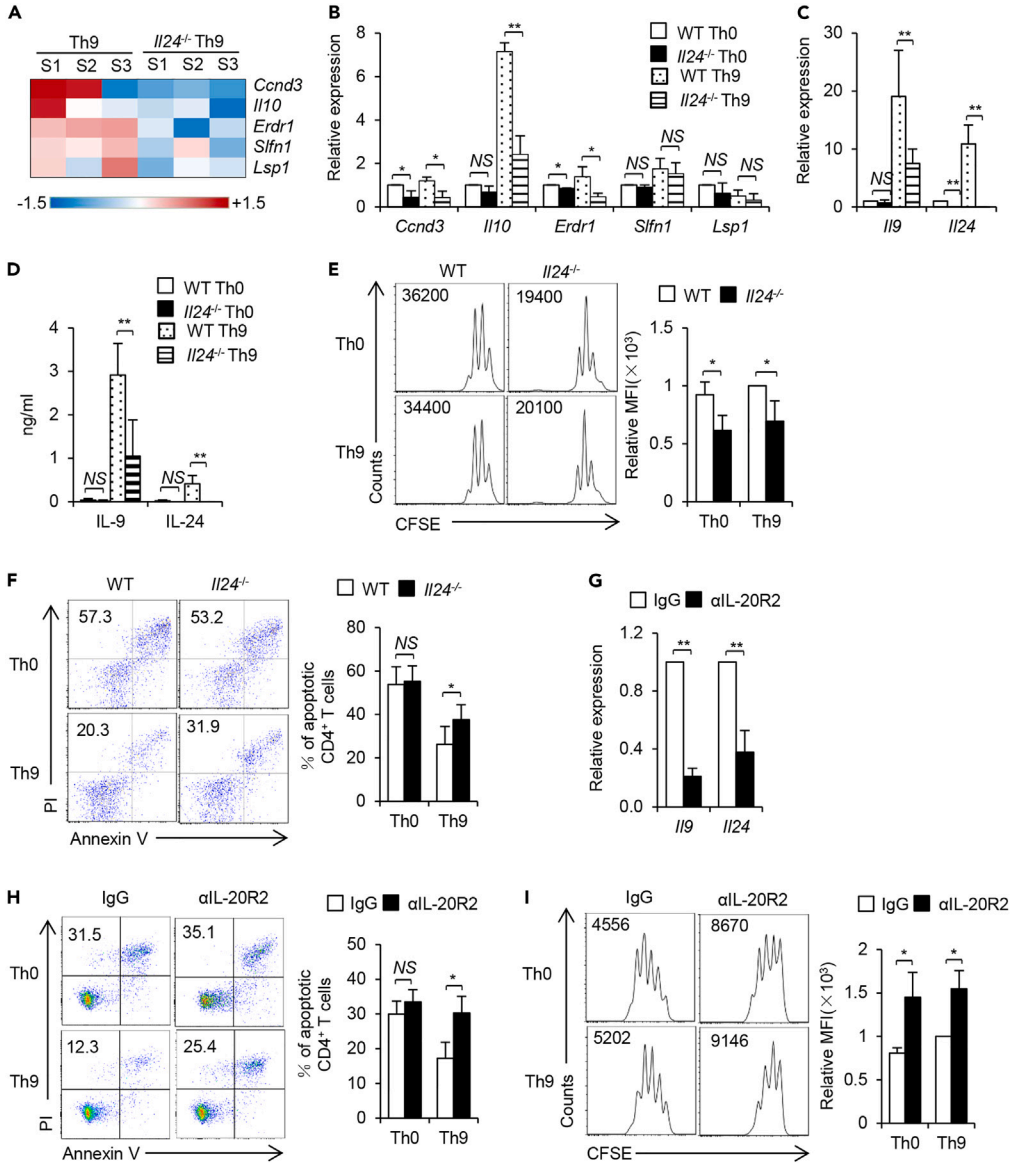

Figure 2. Abrogation of IL-24 increases Th9 cell proliferation and apoptosis *in vitro*

(A) Mouse naive CD4$^+$ T cells from wild type (WT) or *Il24*$^{-/-}$ mice and cultured under Th9 polarizing conditions for 2 days. Cell samples (S1–3) were collected and analyzed by RNA-seq. S1-3: Samples from three independent experiments. The heatmap shows the $\log_2$-fold change of the differentially regulated expression of genes that may be related to T cell survival.

(B–D) Mouse naive CD4$^+$ T cells were isolated from WT or *Il24*$^{-/-}$ mice and cultured under Th0 or Th9 polarizing conditions for 2 days (B) The mRNA levels of the indicated genes were analyzed by qPCR. (C) QPCR analysis of *Il9* and *Il24* in CD4$^+$ T cells. (D) ELISA assessed IL-9 and IL-24 in the cultures.

(E) Naive CD4$^+$ T cells from WT and *Il24*$^{-/-}$ mice were labeled with CFSE and cultured under Th0 or Th9 polarizing conditions for 3 days. CFSE-stained T cells were analyzed by Flow cytometry. Numbers in the histograms represent the fluorescence intensity of CFSE-stained T cells. Right, summarized results of three independent experiments obtained as at left.

(F) Mouse naive CD4$^+$ T cells were cultured under Th0 or Th9 polarizing conditions for 3 days. Cells were analyzed by Flow cytometry. Numbers in the dot plots represent the percentages of apoptotic T cells. Right, summarized results of three independent experiments obtained as at left.

(G and H) Mouse naive CD4$^+$ T cells were cultured under Th9 polarizing conditions with or without the addition of an IL-20R2 neutralizing antibody (αIL-20R2) for 2 (G) or 3 (H) days. (G) qPCR analysis of *Il9* and *Il24* in CD4$^+$ T cells. (H) Cells were

Figure 2. *Continued*

analyzed by Flow cytometry. Numbers in the dot plots represent the percentages of apoptotic T cells. Right, summarized results of three independent experiments obtained as at left.

(I) Naive $CD4^+$ T cells labeled with CFSE were cultured under Th0- or Th9-polarizing conditions with or without the addition of αIL-20R2 for 3 days. Cells were analyzed by Flow cytometry. Numbers in the histograms represent the fluorescence intensity of CFSE-stained T cells. Right, summarized results of three independent experiments obtained as at left. Data are representative of three (E, F, H, I) independent experiments or presented as mean ± SD of three (B-I) independent experiments. *NS*, non-significant; *p < 0.05; **p < 0.01. See also Figure S4.

blot (Figures 1B–1E and S1). We next examined the expression of *Il24* and *Il9* in Th9 cells at different time points. We found that the expression of *Il24* and *Il9* in Th9 cells exhibited a similar time pattern, where they increased on the first day, reached the highest level on the second day, and then slightly decreased on the third day (Figure 1F). The time pattern expression of IL-24 and IL-9 proteins was confirmed by ELISA (Figure 1G).

To further confirm the expression of IL-24 in Th9 cells, we extended Th9 cell cultures to 8 days and collected cells at day 2, 4, 6 and 8. As shown in Figure S2, Th9 cells continuously maintained high expression levels of *Il9* and *Il24* on day 6 and day 8.

IL-4/STAT6 induces IL-24 expression in Th2 cells.[35] We next examined the expression of *Il24*, *Il9* and Th2 cell-related cytokines in Th2 and Th9 cells. As shown in Figure S2, the expression of *Il4*, *Il5*, *Il13*, *Il9* and *Il24* were increased in Th2 cells compared to Th0 cells. And the expression of *Il9* and *Il24* were increased in Th9 cells compared to Th0 cells (Figure S3). However, the expression levels of *Il9* and *Il24* in Th9 cells are significantly higher than those in Th2 cells (Figure S3).

Together, these results demonstrate that IL-24 is highly expressed in Th9 cells.

Abrogation of IL-24 inhibits Th9 cell survival

The differentiation, proliferation and survival of Th9 cells are critical for their persistence and efficacy in immunotherapy. Thus, we next examined the effects of IL-24 on Th9 cell differentiation, proliferation and survival. Naive $CD4^+$ T cells were obtained from wildtype (WT) and *Il24* knockout ($Il24^{-/-}$) mice and cultured under Th9-polarizing conditions for 48 h. RNA-seq analysis were performed. The knockout of IL-24 in Th9 cells had no effects on the expression of Th9-related transcription factors, such as *Spi1*, *Irf4*, *Stata5, Irf1 or Foxo1* (Table S2). However, knockout of IL-24 in Th9 cells induced differential expression of genes: *Ccnd3*, *Il10*, *Erdr1*, *Slfn*, and *Lsp1* (Figure 2A), which are related to T cell proliferation and survival.[36–40] qPCR analysis further confirmed the decreased expression of *Ccnd3*, *Il10* and *Erdr1* in $Il24^{-/-}$ Th9 cells (Figure 2B).

To further exploit the role of IL-24 in Th9 cell differentiation, naive $CD4^+$ T cells were isolated from WT and $Il24^{-/-}$ mice and cultured under Th9-polarizing conditions for 3 days. As expected, $Il24^{-/-}$ Th9 cells failed to produce IL-24 (Figures 2C and 2D). Interestingly, $Il24^{-/-}$ Th9 cells also had lower expression of IL-9 compared to regular Th9 cells (Figures 2C and 2D; Table S3).

To investigate the role of IL-24 in Th9 cell proliferation and survival, Th0 and Th9 cells from WT or $Il24^{-/-}$ mice were generated, and cell proliferation and apoptosis were assessed. As shown in Figure 2E, the knockout of IL-24 in Th9 cells increased cell proliferation in both Th0 and Th9 cells. Though $Il24^{-/-}$ Th0 cells exhibited similar cell apoptosis as compared to WT Th0 cells (Figure 2F), $Il24^{-/-}$ Th9 cells exhibited higher cell apoptosis than WT Th9 cells (Figure 2F). To further confirm the role of IL-24 in Th9 cell survival, we extended Th9 cell cultures to 8 days and counted the cell number at day 2, 4, 6 and 8. As shown in Figure S4, the number of regular Th9 cells continuously increased during Th9 cell cultures and reached a peak on day 6. However, the number of $Il24^{-/-}$ Th9 cells also increased during the first four days of cell cultures and then decreased on day 6 and day 8 (Figure S4). Furthermore, the number of regular Th9 cells is significantly higher than that of $Il24^{-/-}$ Th9 cells on day 6 and 8 during Th9 cell cultures (Figure S4). These results indicate that the abrogation of IL-24 increased Th9 cell apoptosis *in vitro*.

To further explore the role of IL-24 in Th9 cell differentiation, proliferation and survival, we used a blocking antibody against IL-24 receptor IL-20R2 (αIL-20R2) during Th9 cell differentiation. As shown in Figure 2G, αIL-20R2 treatment decreased the expression of both *Il9* and *Il24* in Th9 cells. Furthermore, αIL-20R2

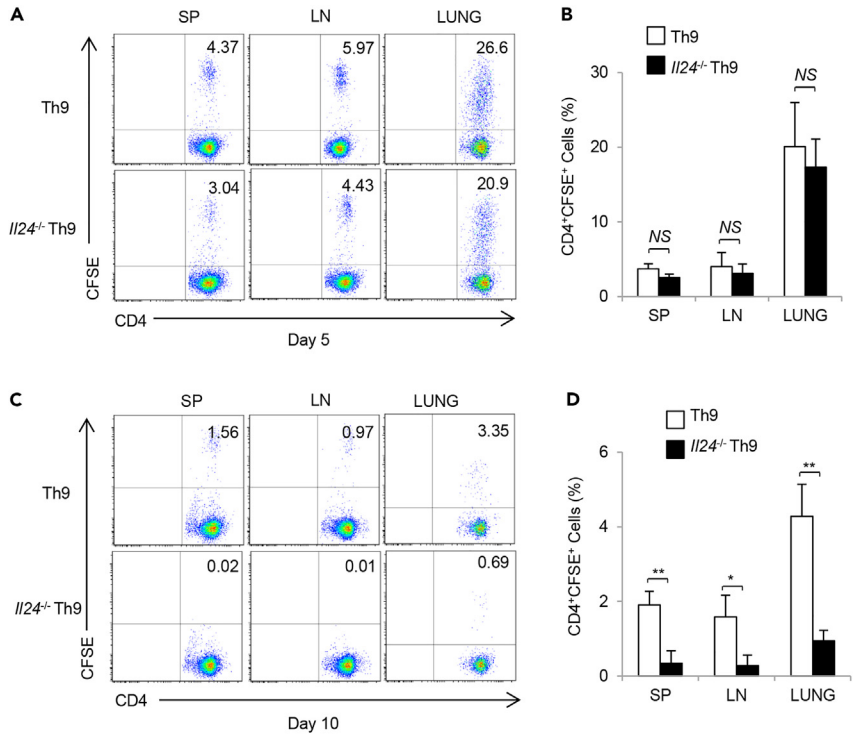

Figure 3. Knockout of IL-24 decreases Th9 cell survival *in vivo*

(A–D) Naive CD4$^+$ T cells from OT-II mice or OT-II-*Il24*$^{-/-}$ mice were cultured under Th9 polarizing conditions for 2 days 5 × 10^6 Th9 cells were labeled with CFSE and injected i.v. into C57BL/6 mice. CFSE-stained Th9 cells from spleen, lymph nodes and lung were analyzed by Flow cytometry at day 5 (A) and day 10 (C). Numbers in the dot plots represent the percentages of CFSE$^+$CD4$^+$ T cells (B and D). Data are representative of three (A and C) independent experiments or presented as mean ± SD of three (B and D) independent experiments. *NS*, non-significant; *p < 0.05; **p < 0.01.

treatment increased Th9 cell apoptosis (Figure 2H). This result is consistent with previous observations that the abrogation of IL-24 increased Th9 cell apoptosis. Interestingly, αIL-20R2 treatment decreased Th9 cell proliferation (Figure 2I). These results indicate the crucial role of IL-24 in Th9 cell survival.

We next analyzed the role of IL-24 in Th9 cell survival *in vivo*. We first generated OT-II-*Il24*$^{-/-}$ mice (Figures S5 and S6). Th9 cells differentiated from OT-II or OT-II-*Il24*$^{-/-}$ mice were labeled with CFSE and adoptively transferred to C57BL/6 mice. CFSE-stained Th9 cells from spleens, lymph nodes (LNs) and lungs were analyzed by Flow cytometry at different time points. We detected significantly lower percentages of IL-24 knockout Th9 (*Il24*$^{-/-}$ Th9) cells in the spleen, LN and lung cells as compared to Th9 cells at day 10 (Figures 3C and 3D), though there was a slightly decrease of *Il24*$^{-/-}$ Th9 cells in the spleen, LNs and lungs cells as compared with Th9 cells at day 5 (Figures 3A and 3B).Together, these results indicate that knockout of IL-24 in Th9 cells decreases the survival of Th9 cells, suggesting that IL-24 contributes to the survival of Th9 cells.

IL-24 is required for Th9 cell-mediated antitumor activity

Naive CD4$^+$ T cells from WT or *Il24*$^{-/-}$ mice were differentiated into Th9 cells. WT or *Il24*$^{-/-}$ Th9 cells were co-cultured with MM cells and separated by Transwell inserts. We first investigated the effects of knockout of IL-24 in Th9 cells on tumor cell proliferation. As shown in Figure 4A, the co-culture of *Il24*$^{-/-}$ Th9 cells resulted in higher expression of Ki67 which is a marker of cell proliferation in 5TGM1 cells as compared to the co-culture of WT Th9 cells. Similar results were obtained when *Il24*$^{-/-}$ Th9 cells were co-cultured with MPC-11 MM cells (Figure 4B). We next examined the effects of the knockout of IL-24 in Th9 cells on tumor cell apoptosis. The co-culture of *Il24*$^{-/-}$ Th9 cells induced less cell apoptosis of 5TGM1 and MPC-11 cells as compared to WT Th9 cells (Figures 4C and 4D). These results indicate that the deficiency of IL-24 abrogated Th9-induced inhibition of tumor cell proliferation and survival, suggesting that Th9 cell-derived IL-24 exerts cytotoxic effects on tumor cells.

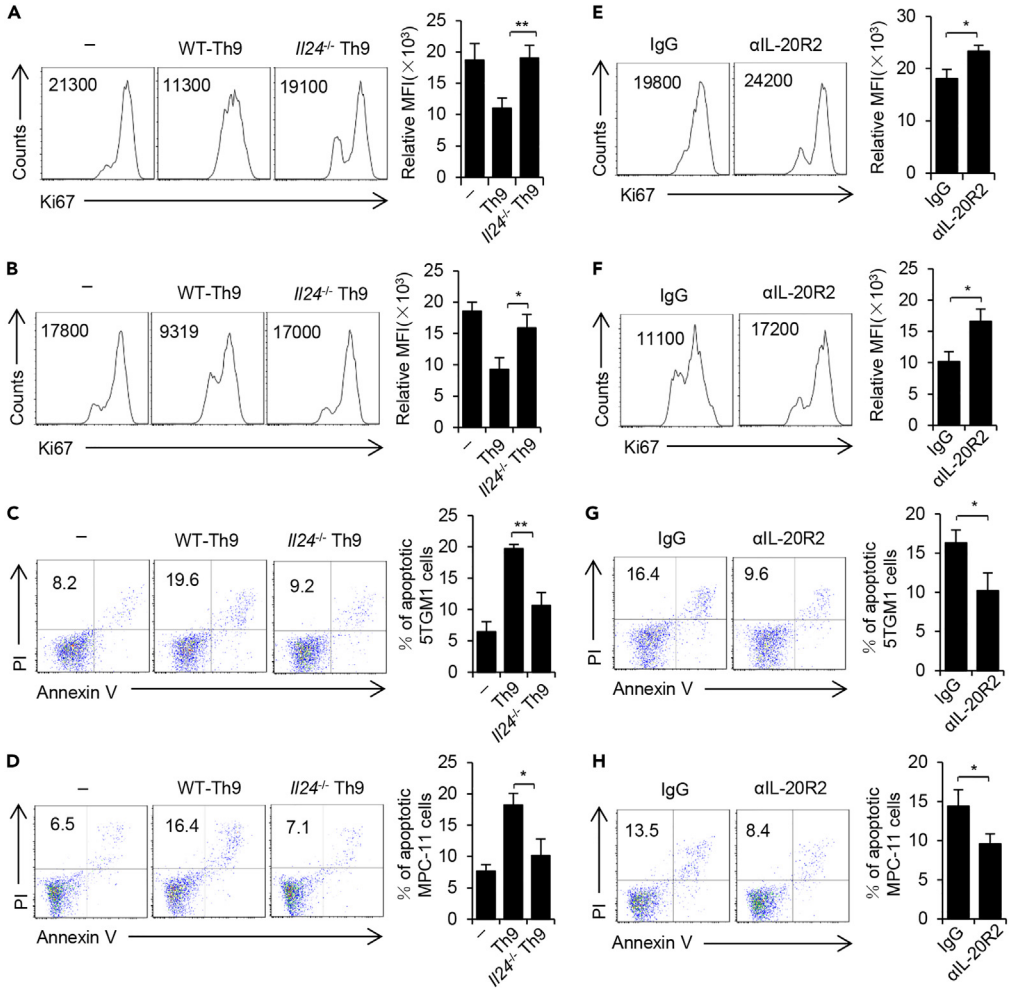

Figure 4. Abrogation of IL-24 decreased Th9 cell antitumor efficacy *in vitro*

(A–D) Mouse naive CD4$^+$ T cells from WT or *Il24*$^{-/-}$ mice were cultured under Th9 polarizing conditions for 24h. Cells were collected and were co-cultured with multiple myeloma (MM) cells in Transwell inserts for another 48h. MM cells without addition of Th9 cells served as controls. (A and B) Flow cytometry analyzed Ki67$^+$ 5TGM1 (A) and MPC-11 (B) cells. Numbers in the dot plots represent the percentages of Ki67$^+$ MM cells. Right, summarized results of three independent experiments obtained as at left. (C and D) Flow cytometry analyzed the apoptosis of 5TGM1 (C) and MPC-11 (D) cells. Numbers in the dot plots represent the percentages of apoptotic MM cells. Right, summarized results of three independent experiments obtained as at left. (E-H) MM cells were pre-treated with αIL-20R2 for 24h. Mouse naive CD4$^+$ T cells from WT mice were cultured under Th9 polarizing conditions for 24h. MM cells were collected and were co-cultured with Th9 cells in Transwell inserts for another 48h.

(E and F) Flow cytometry analyzed Ki67$^+$ 5TGM1 (E) and MPC-11 (F) cells. Numbers in the dot plots represent the percentages of Ki67$^+$ MM cells. Right, summarized results of three independent experiments obtained as at left.

(G and H) Flow cytometry analyzed the apoptosis of 5TGM1 (G) and MPC-11 (H) cells. Numbers in the dot plots represent the percentages of apoptotic MM cells. Summarized results of three independent experiments obtained as at left. Data are representative of three (A-H) independent experiments or presented as mean ± SD of three (A-H) independent experiments. *p < 0.05; **p < 0.01.

To further explore the role of IL-24 in tumor cell proliferation and survival, we used αIL-20R2 to treat MM tumor cells. As shown in Figure 4E, αIL-20R2 treatment increased Ki67 expression in 5TGM1 cells co-cultured withTh9 cells. Similar results were obtained when MPC-11 MM cells were used (Figure 4F). Similarly, αIL-20R2 treatment decreased the apoptosis of MM cells co-cultured withTh9 cells (Figures 4G and 4H). These results indicate that Th9 cell-derived IL-24 mediates tumor regression through IL-24/IL-20R2 signaling pathways.

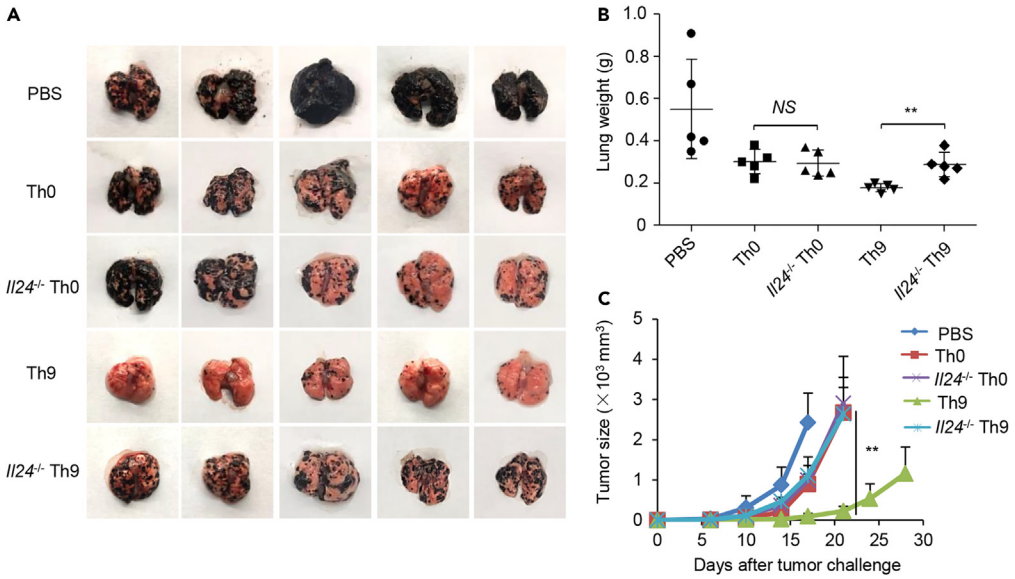

Figure 5. Knockout of IL-24 inhibits the antitumor capability of Th9 cells *in vivo*

Naive CD4$^+$ T cells from OT-II mice or OT-II-*Il24*$^{-/-}$ mice were cultured under Th0 or Th9 polarizing conditions for 2 days. (A and B) After 5 days of i.v. injection of 1 × 10^6 B16-OVA cells into C57BL/6 mice, 5 × 10^6 Th0 or Th9 cells were injected i.v. into tumor-bearing mice. Mice given PBS served as controls. The experiments were performed twice with a total of 10 mice per group (n = 10). (A) Images showing lung tumor development in mice of different treatment groups. (B) Statistics of lung weight in different treatment groups in A.

(C) After 5 days of s.c. injection of 2 × 10^5 B16-OVA cells into C57BL/6 mice, 5 × 10^6 Th0 or Th9 cells were injected i.v. into tumor-bearing mice. Mice given PBS served as controls. Shown are the tumor growth curves. The experiments were performed twice with a total of 10 mice per group (n = 10). Data are presented as mean ± SD of the combined experiments. *NS*, non-significant; **p < 0.01. See also Figure S7.

We next examined the antitumor efficacy of *Il24*$^{-/-}$ Th9 cells. In the lung metastases model, C57BL/6 mice were injected i.v. with B16-OVA melanoma cells and followed by the adoptive transfer of Th9 cells cultured from OT-II mice or OT-II-*Il24*$^{-/-}$ mice. Tumor lung metastasis was examined. As expected, mice that received Th9 cells had lower tumor burden than mice treated with Th0 cells (Figures 5A and 5B). Though *Il24*$^{-/-}$ Th0 cells exerted similar effects on lung tumor burden as compared to Th0 cells (Figures 5A and 5B), The knockout of IL-24 largely abrogated Th9-induced inhibition of tumor lung metastasis (Figures 5A and 5B). Moreover, in the therapeutic B16-OVA tumor model, Th9 cells induced higher tumor growth inhibition than Th0 cells (Figure 5C). *Il24*$^{-/-}$ Th0 cells exerted minor effects on tumor growth, similar to Th0 cells (Figure 5C). However, the deficiency of IL-24 in Th9 cells largely abrogated Th9-induced inhibition on tumor growth (Figure 5C). These results demonstrate that knockout of IL-24 in Th9 cells decreases the antitumor capability of Th9 cells *in vivo*.

Th9 cell-induced antitumor CTLs may be involved in tumor growth inhibition.[7] We next examined CD8$^+$ T cells induced by Th9 cells and *Il24*$^{-/-}$ Th9 cells *in vivo*. As shown in Figure S7A, there was no significant difference in the percentages of CD8$^+$ T cells in spleens, LNs and lungs in mice infused with Th9 cells versus *Il24*$^{-/-}$ Th9 cells, suggesting that CD8$^+$ T cells may not be the major effector in Th9 cell-induced antitumor activity. Granzyme B (GzmB) may be involved in Th9 cell-induced antitumor activity.[6] However, there was no significant difference in the expression of *Gzmb* in Th9 cells versus *Il24*$^{-/-}$ Th9 cells *in vivo* (Figure S7B), though the expression of *Il9* decreased in *Il24*$^{-/-}$ Th9 cells compared to Th9 cells (Figure S7C).

Together, these results demonstrate the important role of IL-24 in Th9 cell-induced antitumor activity, and suggested that IL-24 may be the key antitumor effector of Th9 cells.

Foxo1 contributes to IL-24 expression in Th9 cells

We next explored the transcription factors that control IL-24 expression in Th9 cells. Through gene expression profiling analyses, we found 14 up-regulated and 4 down-regulated transcription factors in Th9 cells compared to Th0 cells (Figure 6A). We hypothesized that IL-24 downstream signaling could not affect

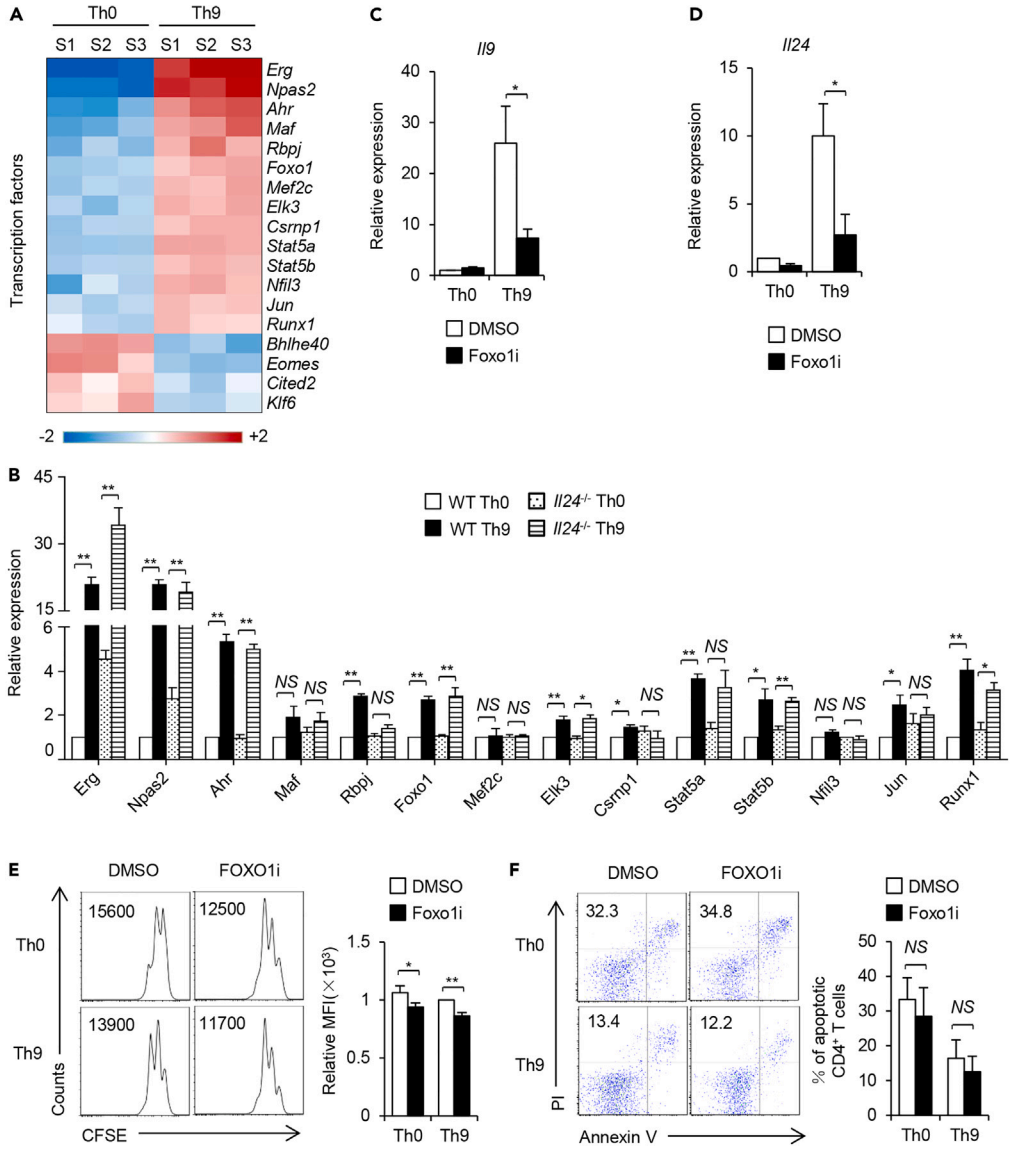

Figure 6. Foxo1 contributes to IL-24 expression in Th9 cells

(A) Another analysis was performed using the RNA-seq data in Figure 1A. The heatmap shows the $\log_2$-fold change of the differentially expressed genes of transcription factors.

(B–D) Mouse naive CD4$^+$ T cells from WT or *Il24*$^{-/-}$ mice and cultured under Th0- or Th9-polarizing conditions for 2 days. The mRNA levels of the indicated transcription factors were analyzed by qPCR (C and D) Mouse naive CD4$^+$ T cells were cultured under Th0 or Th9 polarizing conditions with or without the addition of FOXO1 inhibitor (Foxo1i). qPCR analysis of *Il9* (C) and *Il24* (D) in CD4$^+$ T cells.

(E) Naive CD4$^+$ T cells labeled with CFSE were cultured under Th0- or Th9-polarizing conditions with or without (DMSO) the addition of Foxo1i for 3 days. CFSE-stained T cells were analyzed by Flow cytometry. Numbers in the histograms represent the fluorescence intensity of CFSE-stained T cells. Right, summarized results of three independent experiments obtained as at left.

(F) Mouse naive CD4$^+$ T cells were cultured under Th0- or Th9-polarizing conditions with or without the addition of Foxo1i for 3 days. Annexin V$^+$ PI$^+$ T cells were analyzed by Flow cytometry. Numbers in the dot plots represent the percentages of Annexin V$^+$ PI$^+$ T cells. Right, summarized results of three independent experiments obtained as at left. Data are representative of three (E, F) independent experiments or presented as mean ± SD of three (B-F) independent experiments. *NS*, non-significant; *p < 0.05; **p < 0.01.

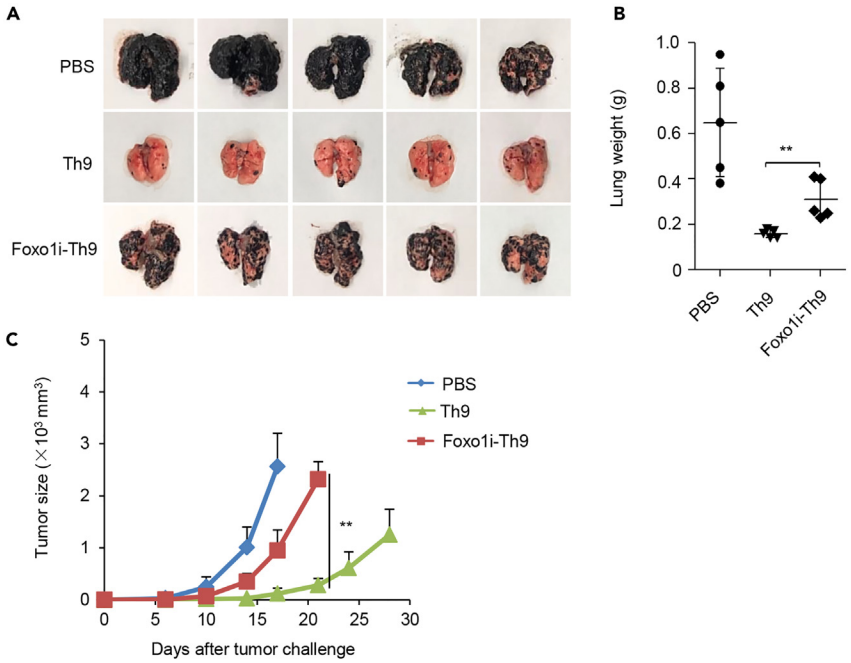

Figure 7. Inhibition of Foxo1 decreases the antitumor efficacy of Th9 cells *in vivo*

Naive $CD4^+$ T cells from OT-II mice or OT-II-*Il24*$^{-/-}$ mice were cultured under Th9-polarizing conditions for 2 days with or without the addition of Foxo1i.

(A and B) After 5 days of i.v. injection of 1 × 10^6 B16-OVA cells into C57BL/6 mice, 5 × 10^6 Th9 cells were injected i.v. into tumor-bearing mice. Mice given PBS served as controls. The experiments were performed twice with a total of 10 mice per group (n = 10). (A) Images showing lung tumor development in mice of different treatment groups. (B) Statistics of lung weight in different treatment groups in A.

(C) After 5 days of s.c. injection of 2 × 10^5 B16-OVA cells into C57BL/6 mice, 5 × 10^6 Th9 cells were injected i.v. into tumor-bearing mice. Mice given PBS served as controls. The experiments were performed twice with a total of 10 mice per group (n = 10). Shown are the tumor growth curves. Data are presented as mean ± SD of the combined experiments. **p < 0.01. See also Figure S8.

the expression of these transcription factors. So we chose genes which have increased expression in both Th9 and *Il24*$^{-/-}$ Th9 cells compared to their Th0 cell controls. QPCR analysis showed that the expression of *Erg*, *Npas2*, *Ahr*, *Foxo1*, *Elk3*, *Stat5b*, and *Runx1* were increased in Th9 and *Il24*$^{-/-}$ Th9 cells (Figure 6B), suggesting that these transcription factors may be involved in the expression of IL-24 in Th9 cells.

We found that the expression of IL-24 and IL-9 in Th9 cells exhibited the similar time pattern, suggesting that the expression of IL-24 and IL-9 may be regulated by some common transcription factors in Th9 cells. Foxo1 is shown to be involved in the differentiation of Th9 cells and the expression of IL-9.[5] To investigate the role of Foxo1 in the expression of IL-24 in Th9 cells, we treated Th9 cells with AS1842856 (a Foxo1 inhibitor, Foxo1i). As shown in Figures 6C and 6D, Foxo1i treatment decreased the expression of both *Il9* and *Il24* in Th9 cells. Furthermore, Foxo1i treatment increased Th9 cell proliferation (Figure 6E), but had minor effects on Th9 cell apoptosis (Figure 6F).

To further validate the role of Foxo1in the antitumor activity of Th9 cells, we examined the efficacy of Foxo1i-treated Th9 (Foxo1i-Th9) cells in the inhibition of lung tumor metastasis. As shown in Figures 7A and 7B, Foxo1i-Th9 cells increased the lung tumor burden in mice as compared with regular Th9 cells. Moreover, Foxo1i-Th9 cells also exhibited less inhibition on B16-OVA tumor growth as compared to regular Th9 cells (Figure 7C). To examine the role of IL-24 in the antitumor effects induced by Foxo1i-Th9 cells, recombinant IL-24 was used. As shown in Figure S8, the addition of IL-24 partially restored the antitumor efficacy of Foxo1i-Th9 cells.

Taken together, these results indicate that Foxo1 is a common transcription factor which contributes to the expression of IL-24 and IL-9 in Th9 cells.

DISCUSSION

Tumor-specific Th9 cells exhibit powerful antitumor capacity.[18] However, the underlying mechanism of Th9 cell-mediated antitumor activity still remains elusive. In this study, we found high expression levels of IL-24 in Th9 cells. In addition, the expression of IL-24 and IL-9 in Th9 cells exhibited a similar time pattern. We found that the knockout of IL-24 abrogated Th9-induced inhibition of tumor cell proliferation and survival. More importantly, in a mouse tumor model, adoptive immunotherapy with IL-24-knockout Th9 cells had less antitumor effects than conventional Th9 cells. Multiple mechanisms may be involved in IL-24-induced antitumor activity of Th9 cells. Secreted IL-24 protein suppresses tumor cell growth and induces tumor cell apoptosis.[29,30] In this study, we also found that knockout of IL-24 in Th9 cells abrogates Th9-induced antitumor activity in the Transwell cultures, indicating that Th9-derived IL-24 exerts directly cytotoxic effects on tumor cells. It has been shown that the antitumor efficacy of Th9 cells depends on IL-9.[7,18] In this study, we found that the knockout of IL-24 in Th9 cells decreases Th9 cell expression of IL-9, which may result in decreased antitumor efficacy of Th9 cells.

T cell survival is crucial for adoptive tumor immunotherapy.[18] In this study, we found that the knockout of IL-24 in Th9 cells decreases Th9 cell survival, indicating that IL-24 contributes to Th9 cell survival. Thus, our results demonstrate the crucial role of IL-24 in Th9 cell-mediated antitumor activity. The identification of factors that can increase IL-24 expression in Th9 cells may greatly improve the clinical efficacy in Th9 cell-mediated tumor therapy.

IL-24 induces proliferation inhibition and cell apoptosis in a variety of tumors, but has minor effects on cell proliferation and cell apoptosis in normal cells.[41–44] In this study, we found that knockout of IL-24 in Th9 cells increases both proliferation and apoptosis of Th9 cell *in vitro*. However, cell count data showed that knock out of IL-24 in Th9 cells resulted in a decrease in cell numbers, suggesting the dominant role of IL-24 in Th9 cell survival. Moreover, knockout of IL-24 in Th9 cells reduced the survival of Th9 cells *in vivo*, suggesting that IL-24 contributes to the survival of Th9 cells *in vivo*. Th9 cell survival is crucial for Th9 cell mediated-tumor immunotherapy. Therefore, it is necessary to further investigate the underlying mechanism of IL-24-induced Th9 cell survival.

Our data show a similar expression time pattern of IL-24 and IL-9 in Th9 cells, suggesting that the expression of IL-24 and IL-9 may be regulated by some common transcription factors in Th9 cells. Foxo1 is shown to be involved in Th9 cell differentiation and IL-9 expression.[5] Our data also show that Foxo1 is highly expressed in Th9 cells. Furthermore, Foxo1 inhibitor treatment decreased the expression of both IL-24 and IL-9 in Th9 cells, indicating that Foxo1 is a transcription factor that contributes to the expression of both IL-24 and IL-9 in Th9 cells. Thus, our data identified Foxo1 as a transcription factor that contributes to IL-24 expression.

IL-9 enhances T cell survival.[15] Our data show that IL-24 contributes to Th9 cell survival. We also show that Foxo1 contributes the expression of both IL-24 and IL-9 in Th9 cells. However, in this study, we found that Foxo1 inhibition exhibits minor effects on Th9 cell survival. These observations indicated that Foxo1 may regulate some pathways that counteract the effect of IL-24 and IL-9 on Th9 cell survival. Further studies will be necessary to explore the underlying mechanism by which Foxo1 regulates Th9 cell survival.

IL-24 may promote Th9 cell survival through intracellular and/or extracellular forms. In this study, our data show that the addition of a blocking antibody against IL-24 cell surface receptor IL-20R2 decreased Th9 cell survival. This result demonstrates that IL-24 contributes to Th9 cell survival through extracellular protein form, at least in part.

Th9 cell-derived IL-24 may exert antitumor activity by directly targeting on tumor cells and/or by activating some other antitumor mechanisms. Our data show that the addition of a blocking antibody against IL-20R2 reduced Th9 cell antitumor efficacy, demonstrating the direct antitumor efficacy of Th9 cell-derived IL-24. $CD8^+$ T cells and GzmB may be involved in Th9 cell-induced antitumor activity.[6,7] However, our data show that there was no significant difference in the induction of $CD8^+$ T cells by Th9 cells versus IL-24 knockout Th9 cells *in vivo*. And there was also no significant difference in the expression of GzmB in Th9 cells versus IL-24 knockout Th9 cells *in vivo*. Based on these observations, Th9 cell-derived IL-24 may exert antitumor efficacy mainly through the directly targeting on tumor cells.

Ulrich reported that the expression of IL-24 was continuously increased in Th9 cells from day 0 to day 10, but the expression of IL-9 in Th9 cells reached the peak at day 5 and then decreased during the 15-day Th9 cell cultures.[34] However, our data show that Th9 cells continuously expressed high level of IL-24 and IL-9 through to the 8-day Th9 cell cultures. The mechanisms underlying the different expression trends of IL-9 in Th9 cells were not determined. However, in Ulrich's study, the cells were cultured under Th9-polarizing conditions for 3 rounds and anti-CD3-coated plates, anti-CD28, IL-4 and TGF-β1 were used in each round of cell culture. However, in our Th9 cell cultures, we added anti-CD3, anti-CD28, IL-4 and TGF-β1 only on day 0 and just changed the medium without adding any cytokines on day 4. These observations suggest that the repeated addition of Th9-polarizing cytokines IL-4 and TGF-β1 may inhibit IL-9 expression in Th9 cells.

The antitumor effects of Th9 cells depend on not only the expression of cytotoxic molecules, such as IL-24, but also the persistence of Th9 cells *in vivo*. However, the molecular mechanisms underlying IL-24 expression and Th9 cell survival may be different. IL-24 promotes Th9 cell survival but induces tumor cell apoptosis. The underlying mechanisms are still not defined. Therefore, further studies are necessary and these studies may further increase the antitumor efficacy of Th9 cells in tumor immunotherapy.

In summary, our study demonstrates that IL-24 is highly expressed in Th9 cells. The knockout of IL-24 in Th9 cells increases both the proliferation and apoptosis of Th9 cells *in vitro*. The adoptive transfer of IL-24 knockout Th9 cells induced less antitumor effects than regular Th9 cells in mouse tumor model. Foxo1 regulates IL-24 expression in Th9 cells. Inhibition of Foxo1 decreases IL-24 expression in Th9 cells and inhibits the antitumor efficacy of Th9 cells *in vivo*. Our results identify IL-24 as a powerful antitumor effector of Th9 cells and provide a target in Th9 cell-mediated tumor therapy.

Limitations of the study

In this study, we discovered that IL-24 is essential for Th9 cell-mediated antitumor efficacy. We found that IL-24-deficient Th9 cells lose their antitumor capability *in vitro* and *in vivo*. Furthermore, we found that the regular Th9 cells but not IL-24-deficient Th9 cells exhibit antitumor effects in the Transwell cultures, suggesting that some soluble molecules (very possibly IL-24) executed the Th9 cell-mediated antitumor efficacy. To verify the direct role of IL-24 in Th9 cell antitumor immunotherapy, we need mouse IL-24 neutralizing antibodies. Unfortunately, no commercial IL-24 neutralizing antibodies are available. Further studies are needed to verify the direct role of IL-24 in Th9 cell-mediated antitumor activities.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

Conceptualization, S.W. and S.G.; Methodology, Data curation, S.W. and J.C.; Validation, J.C and Y.Z.; Investigation, J.C., Y.Z., H.Z., M.Z., H.D., and T.Q.; Writing–original draft, J.C.; Writing–review and editing, S.W.; Visualization, J.C.; Supervision, S.W.; Project Administration, J.C.; Funding acquisition, S.W. and J.C.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CD3e	eBioscience	Cat#16-0031-86; RRID: AB_468849
CD28	eBioscience	Cat#16-0281-86; RRID: AB_468923
PE Rat Anti-Mouse CD3	BD Biosciences	Cat# 555275; RRID: AB_395699
APC Rat Anti-Mouse CD4	BD Biosciences	Cat# 553051; RRID: AB_398528
PerCP-Cy5.5 Rat Anti-Mouse CD8a	BD Biosciences	Cat#551162; RRID: AB_394081
Alexa Fluor 700 Rat Anti-Mouse CD62L	BD Biosiences	Cat# 560517; RRID: AB_1645210
FITC Rat Anti-Mouse CD25	BD Biosciences	Cat#553072; RRID:AB_394603
FITC IL24 antibody	biorbyt	Cat#orb188601
BV421 anti-mouse Ki-67	BioLegend	Cat#652411; RRID: AB_2562663
IL-20R2 antibody	eBiosciences	Cat#14-1206-82; RRID: AB_1944396
IL24 Polyclonal Antibody	Invitrogen	Cat#PA5-109854; RRID: AB_2855265
β-Actin (13E5) Rabbit mAb	CST	Cat#4970S; RRID: AB_2223172
HRP-conjugated Affinipure Goat Anti-Rabbit IgG	proteintech	Cat#SA00001-2; RRID: AB_2722564
Chemicals, peptides, and recombinant proteins		
RPMI-1640 medium	BI	Cat#01-100-1ACS
Fetal bovine serum	Gibco	Cat#10091-148
Penicillin-Streptomycin solution	Hyclone	Cat#SV30010
Recombinant mouse IL-4 protein	R&D Systems	Cat#404-ML-010;
Recombinant human TGF-β	R&D Systems	Cat#240-B-002
Recombinant mouse IL-24 protein	R&D Systems,	Cat#7807-ML-010
AS1842856	MedChemExpress	Cat#HY-100596; CAS: 836620-48-5
CellTrace CFSE	Invitrogen	Cat#C34557
TRIzol Reagent	Invitrogen	Cat#15596–026
All-in-One First-Strand cDNA Synthesis SuperMix	TransGen Biotech	Cat#AT341-02
FastStart Universal SYBR Green Master	Roche	Cat#04913914001
RIPA Buffer	CST	Cat#9806
Protease/Phosphatase inhibitor cocktail	CST	Cat#5872
Critical commercial assays		
EasyPure RNA Kit	TransGen Biotech	Cat#ER101-01
IL-9 ELISA MAX Deluxe	BioLegend	Cat#442704
IL-24 DuoSet ELISA	R&D System	Cat#DY2786-05
BD Cytofix/Cytoperm™ Plus Fixation/ Permeabilization Solution Kit with BD GolgiStop™	BD Biosiences	Cat#554715; RRID: AB_2869009
FITC Annexin V Apoptosis Detection Kit	BD Biosciences	Cat#556547; RRID: AB_2869082
BCA protein assay kit	Thermo	Cat#23227
Immobilon Western Chemiluminescent HRP Substrate	Millipore	Cat#WBKLS0050

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited data		
RNA-seq raw data	NCBI Sequence Read Archive (SRA) repository	SRA: PRJNA930048
Original western blot images	This paper	see Figure S1
Experimental models: Cell lines		
MPC-11	Qing Yi	N/A
B16-OVA	Qing Yi	N/A
5TGM1	Qing Yi	N/A
Experimental models: Organisms/strains		
C57BL/6 mice	The Jackson Laboratory	JAX:000664
OT-II (C57BL/6-Tg(TcraTcrb)425Cbn/J)	The Jackson Laboratory	JAX: 004194
Il24$^{-/-}$ (B6N(Cg)-*Il24*$^{tm1.1(KOMP)Vlcg}$/2J)	The Jackson Laboratory	N/A
Oligonucleotides		
Primer pairs for qPCR	This paper	see in Table S3
Software and algorithms		
GraphPad Prism version 5.0	GraphPad	N/A
FlowJo software version X	FlowJo	N/A
Image Lab	Image	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Siqing Wang (siw1970@yahoo.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- The microarray data in this study is stored in the NCBI Sequence Read Archive (SRA) repository under accession number PRJNA930048 and the accession number is listed in the key resources table. The original western blot images were shown in Figure S1.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Mice

C57BL/6, OT-II (C57BL/6-Tg(TcraTcrb)425Cbn/J) and *Il24*$^{-/-}$ (B6N(Cg)-Il24$^{tm1.1(KOMP)Vlcg}$/2J) mice were purchased from the Jackson Laboratory. OT-II- *Il24*$^{-/-}$ mice were examined and confirmed (Figures S1 and S2 in supplemental information). All mice were bred and maintained under specific pathogen-free conditions at the First Hospital Animal Center of Jilin University. Mice aged from 6 to 8 weeks were used for the experiment. An equal number of males and females were assigned to the experimental group though a random assignment process for *in vivo* experiments. And females were used for *in vitro* experiments. All animal experiments were approved by the Animal Ethical Committee of First Hospital of Jilin University ((Permit protocol: 2019-039).We have complied with all relevant ethical regulations for animal testing and research.

Cell lines

MPC-11 and 5TGM1 multiple myeloma (MM) cell lines and B16-OVA melanoma cell line were provided by Qing Yi (Lerner Research Institute, Cleveland Clinic, Cleveland). All cells were identified by short tandem repeat profiling and were free of mycoplasma. Cells were cultured in RPMI 1640 medium (BI) supplemented with 10% heat-inactivated fetal bovine serum (FBS, ExCell), 1% Penicillin-Streptomycin solution (Hyclone). Cells were cultured in a standard (37°C, 5% CO_2) incubator.

METHOD DETAILS

In vitro Th9 cell differentiation

Naive $CD4^+$ T cells ($CD4^+CD25^-CD62L^{hi}$) were isolated from mouse spleen cells by fluorescence activated cell sorter (FACS). Naïve $CD4^+$ T cells were cultured in an environment with plate-bound anti-CD3 (2 μg/mL), soluble anti-CD28 (2 μg/mL) and Th9-polarizing cytokines IL-4 (10 ng/mL) and TGF-β (3 ng/mL). Cells cultured without the addition of IL-4 and TGF-β were used as Th0 cells. In some cell cultures, AS1842856 (a Foxo1 inhibitor, 100 nM) or IL-20R2 antibody (αIL-20R2, 0.5ug/ml) was added. After 2 or 3 days of culture, cells were collected and analyzed by Flowcytometry and/or qPCR. And culture supernatants were harvested and analyzed by ELISA.

In vitro Th2 cell differentiation

Naive $CD4^+$ T cells ($CD4^+CD25^-CD62L^{hi}$) were isolated from mouse spleen cells by FACS. Naïve $CD4^+$ T cells were cultured in an environment with plate-bound anti-CD3 (2 μg/mL), soluble anti-CD28 (2 μg/mL) and Th2-polarizing cytokines IL-4 (10 ng/mL).

RNA sequencing (RNA-Seq)

Naïve $CD4^+$ T cells from C57BL/6 or IL-$24^{-/-}$ mice were cultured under Th0- or Th9-polarizing conditions for 48h. Cells were collected and stored in Trizol reagent (Invitrogen) at -80°C. RNA-Seq was performed by Sangon Biotech (Shanghai, China) with Illumina Xten. Detailed information is as follows:

RNA isolation

Total RNA was extracted using the Total RNA Extractor (Trizol) kit (B511311, Sangon, China) according to the manufacturer's protocol, and treated with RNase-free DNase I to remove genomic DNA contamination. RNA integrity was evaluated with a 1.0% agarose gel. Thereafter, the quality and quantity of RNA were assessed using a NanoPhotometer ® spectrophotometer (IMPLEN, CA, USA) and a Qubit® 2.0 Flurometer (Invitrogen). The high quality RNA samples were subsequently submitted to the Sangon Biotech (Shanghai) Co., Ltd. for library preparation and sequencing.

Library preparation and sequencing

A total amount of 1 μg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using VAHTSTM mRNA-seq V2 Library Prep Kit for Illumina® following manufacturer's recommendations and index codes were added to attribute sequences to each sample. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in VAHTSTM First Strand Synthesis Reaction Buffer (5X). First strandcDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNase H-). Second strand cDNA synthesis was subsequently performed using DNA polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3′ ends of DNA fragments, adaptor was ligated to prepare for library. In order to select cDNA fragments of preferentially 150~200 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). Then 3 μLUSER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37°C for 15 min followed by 5 min at 95°C before PCR. Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. At last, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system. The libraries were then quantified and pooled. Paired-end sequencing of the library was performed on the NovaSeq sequencers (Illumina, San Diego, CA).

Data assessment and quality control

FastQC (version 0.11.2) was used for evaluating the quality of sequenced data. Raw reads were filtered by Trimmomatic (version 0.36) according to several steps: 1) Removing adaptor sequence if reads contains; 2) Removing low quality bases from reads 3′ to 5′ (Q < 20); 3) Removing low quality bases from reads 5′ to 3′ (Q < 20); 4) Using a sliding window method to remove the base value less than 20 of reads tail (window size is 5 bp); 5) Removing reads with reads length less than 35 nt and its pairing reads. And the remaining clean data was used for further analysis.

Alignment with reference genome

Clean reads were mapped to the reference genome by HISAT2 (version 2.0) with default parameters. RSeQC (version 2.6.1) was used to statistics the alignment results. The homogeneity distribution and the genome structure were checked by Qualimap (version 2.2.1). BEDTools (version 2.26.0) was used to statistical analysis the gene coverage ratio.

Quantitative polymerase chain reaction (qPCR)

Cellular RNA was extracted by using an EasyPure RNA Kit (TransGen Biotech). And cDNA was synthesized by using an All-in-One First-Strand cDNA Synthesis SuperMix (TransGen Biotech). The expression of *Il9, Il24, Il4, Il5, Il13,Erg, Npas2, Id1, Ahr, Maf, Rbpj, Foxo1, Mef2c, Elk3, Csrnp1, Stat5a, Stat5b, Nfil3, Jun, Runx1, Gzmb, Il10, Ccnd3, Erdr1, Slfn1, Lsp1* by Th cells were analyzed with SYBR Green real-time PCR (Applied Biosystems). Gene expression was normalized to the housekeeping gene *Gapdh*. Gene primers are shown in Table S3 in supplemental information.

Enzyme-linked immunosorbent assay (ELISA)

Cell culture supernatants were harvested and analyzed by ELISAs to determine the concentrations of IL-9 with Mouse IL-9 ELISA MAX Deluxe (BioLegend) and IL-24 with Mouse IL-24 DuoSet ELISA (R&D System) according to the manufacture's protocol.

Flow cytometry

PE-, APC-, PerCP-Cy5.5-, FITC-, or Alex Fluor 700 -conjugated mAbs against CD3 (cat #: 555275; RRID: AB_395699), CD4 (cat #: 553051; RRID: AB_398528), CD8 (cat #: 551162; RRID: AB_394081), CD25 (cat #: 553072; RRID: AB_394603) and CD62L (cat #: 560517; RRID: AB_1645210) were purchased from BD Biosciences. FITC-conjugated mAbs against IL-24 (cat #: orb188601) were purchased from Biorbyt. BV421-conjugated mAbs against Ki67 (cat #: 652411; RRID: AB_2562663) were purchased from BioLegend. Intracellular staining and intranuclear staining were performed by using a Cytofix/Cytoperm kit (BD Biosciences) or a Transcription Factor Fixation/Permeabilization Buffer Set (BioLegend). Cell viability was measured by using a FITC Annexin V Apoptosis Detection Kit (BD Biosciences). Cell proliferation was measured by using a CFSE Cell Proliferation Kit (Invitrogen). Cells were acquired and analyzed by a BD LSRFortessa™ cytometer.

Western-blot

Cells were lysed by RIPA Buffer (CST, cat#:9806) containing protease inhibitors (CST, cat#: 5872) and the concentrations of total protein were quantified by a BCA protein assay kit (Thermo, cat#: 23227). Precipitate of protein by using metal bath for 10 min at 95°C. Protein was separated by 15% SDS-PAGE gel and transferred onto PVDF membranes (Millipore). The membranes were blocked with blocking buffer (5% skim milk in PBST) for 1 h and then were incubated with primary antibodies at 4°C overnight. The primary antibodies included anti-IL-24 (1:3000; Invitrogen, cat#: PA5-109854), anti-β-actin (1:1000; CST, cat #: 4970S). After washing with PBST, HRP-conjugated goat-anti-rabbit secondary antibodies (1:3000; proteintech, Cat#: SA00001-2) were used to incubate the membranes for 1 h at room temperature. Finally, the membranes were washed again and the signals were visualized by Chemiluminescent HRP Substrate (Millipore).

Adoptive tumor immunotherapy

C57BL/6 mice were injected subcutaneously (s.c.) with B16-OVA cells (2×10^5 cells/mouse). Naïve $CD4^+$ T cells from OT-II mice or OT-II-*Il24*$^{-/-}$ mice were cultured under Th0- or Th9-polarizing conditions for 2 days. In some experiments, AS1842856 were given during Th9 cell cultures. On the 5th day after tumor injection, mice were randomly assigned to receive intravenous (i.v.) injection of Th0 or Th9 cells (5×10^6 cells/mouse). In some experiments, mice were given Recombinant Mouse IL-24 (5ug/ per mouse; R&D Systems, 7807-ML-010) along with Th9 cell therapy. Mice treated with PBS were used as controls. The

development of the tumor was monitored over time. When the tumor diameter reached 1.5 to 2 cm, the mice were executed.

To test the lung metastasis of tumor, C57BL/6 mice were injected i.v. with B16-OVA cells (1×10^6 cells/mouse). Naïve $CD4^+$ T cells from OT-II mice or OT-II-*Il24*$^{-/-}$ mice were cultured under Th0- or Th9-polarizing conditions for 2 days. On the 5^{th} day after tumor injection, mice were randomly assigned to receive i.v. injection of Th0 or Th9 cells (5×10^6 cells/mouse). Mice treated with PBS were used as controls. In some experimental groups, mice were simultaneously given Foxo1i. On the 16^{th} or 17^{th} day after tumor injection, mice were sacrificed and lung metastasis of the tumor was detected.

In vivo functional tests for Th9 cells

C57BL/6 mice were injected i.v. with B16-OVA cells (1×10^6 cells/mouse). Naïve $CD4^+$ T cells from OT-II mice or OT-II-*Il24*$^{-/-}$ mice were cultured under Th9-polarizing conditions for 2 days. On the 5^{th} day after tumor injection, mice were randomly assigned to receive i.v. injection of CFSE-labeled Th9 cells (5×10^6 cells/mouse). Mice injected with PBS served as controls. On day 5 after the Th9 cell immunization, total cells from spleens, lymph nodes and lungs were analyzed by Flowcytometry and $CFSE^+$ Th9 cells were isolated by FACS and analyzed by qPCR.

QUANTIFICATION AND STATISTICAL ANALYSIS

Graphpad software version 5.0 or Microsoft Excel was used for data analysis and graphs. The Student's *t*-test (2 groups) and ANOVA (>= 3 groups) were used to compare various experimental groups. Data were presented as mean and standard deviations (mean $\pm$ SD). A *P* value of less than 0.05 was considered significant. The values of significant levels were set as $*p < 0.05$, $**p < 0.01$and *NS* means not significant.