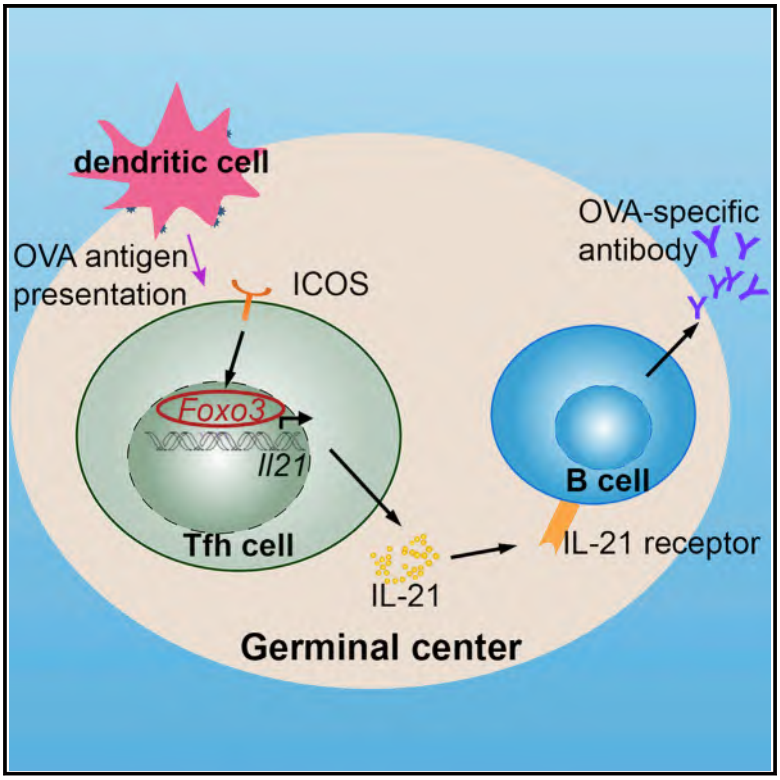
Cell Reports

Foxo3 Promotes the Differentiation and Function of Follicular Helper T Cells

Graphical Abstract



Authors

Haiyu Qi, Dan Tian, Mingyang Li, ..., Qing Ge, Ting Duan, Dong Zhang

Correspondence

duanting888888@126.com (T.D.), zhangd@ccmu.edu.cn (D.Z.)

In Brief

Qi et al. show that Foxo3 promotes Tfh differentiation and IL-21 secretion. Loss of Foxo3 reduces the ability of Tfhs to induce GC B cells and impairs the differentiation and function of B cells. Modulating Foxo3 activity may be beneficial for enhancing or preventing antibody-mediated immune responses.

Highlights

- Foxo3 is a key molecule in Tfh cell differentiation
- Foxo3 affects intrinsic function of Tfh-inducing GC B cells
- Foxo3 binds to the IL-21 promoter and regulates IL-21 secretion of $CD4^+$ T cells

Qi et al., 2020, Cell Reports *31*, 107621
May 12, 2020
https://doi.org/10.1016/j.celrep.2020.107621

CellPress

Cell Reports

CellPress
OPEN ACCESS

Report

Foxo3 Promotes the Differentiation and Function of Follicular Helper T Cells

Haiyu Qi,[1,9] Dan Tian,[2,3,4,9] Mingyang Li,[2,3,4] Chunpan Zhang,[2,3,4] Hua Jin,[2,3,4] Liwei Liu,[5] Xinyan Zhao,[5] Linlin Ma,[6] Weijia Zhao,[7] Qing Ge,[7] Ting Duan,[1,*] and Dong Zhang[2,3,4,5,8,10,*]

[1]Department of Rheumatology and Immunology, Beijing Friendship Hospital, Capital Medical University, Beijing 100050, China
[2]Immunology Research Center for Oral and Systemic Health, Beijing Friendship Hospital, Capital Medical University, Beijing 100050, China
[3]Beijing Clinical Research Institute, Beijing 100050, China
[4]Beijing Key Laboratory of Tolerance Induction and Organ Protection in Transplantation, Beijing 100050, China
[5]National Clinical Research Center for Digestive Diseases, Beijing 100050, China
[6]Department of Urology, Beijing Friendship Hospital, Capital Medical University, Beijing 100050, China
[7]Department of Immunology, School of Basic Medical Sciences, Peking University, Beijing 100191, China
[8]General Surgery Department, Beijing Friendship Hospital, Capital Medical University, Beijing 100050, China
[9]These authors contributed equally
[10]Lead Contact
*Correspondence: duanting888888@126.com (T.D.), zhangd@ccmu.edu.cn (D.Z.)
https://doi.org/10.1016/j.celrep.2020.107621

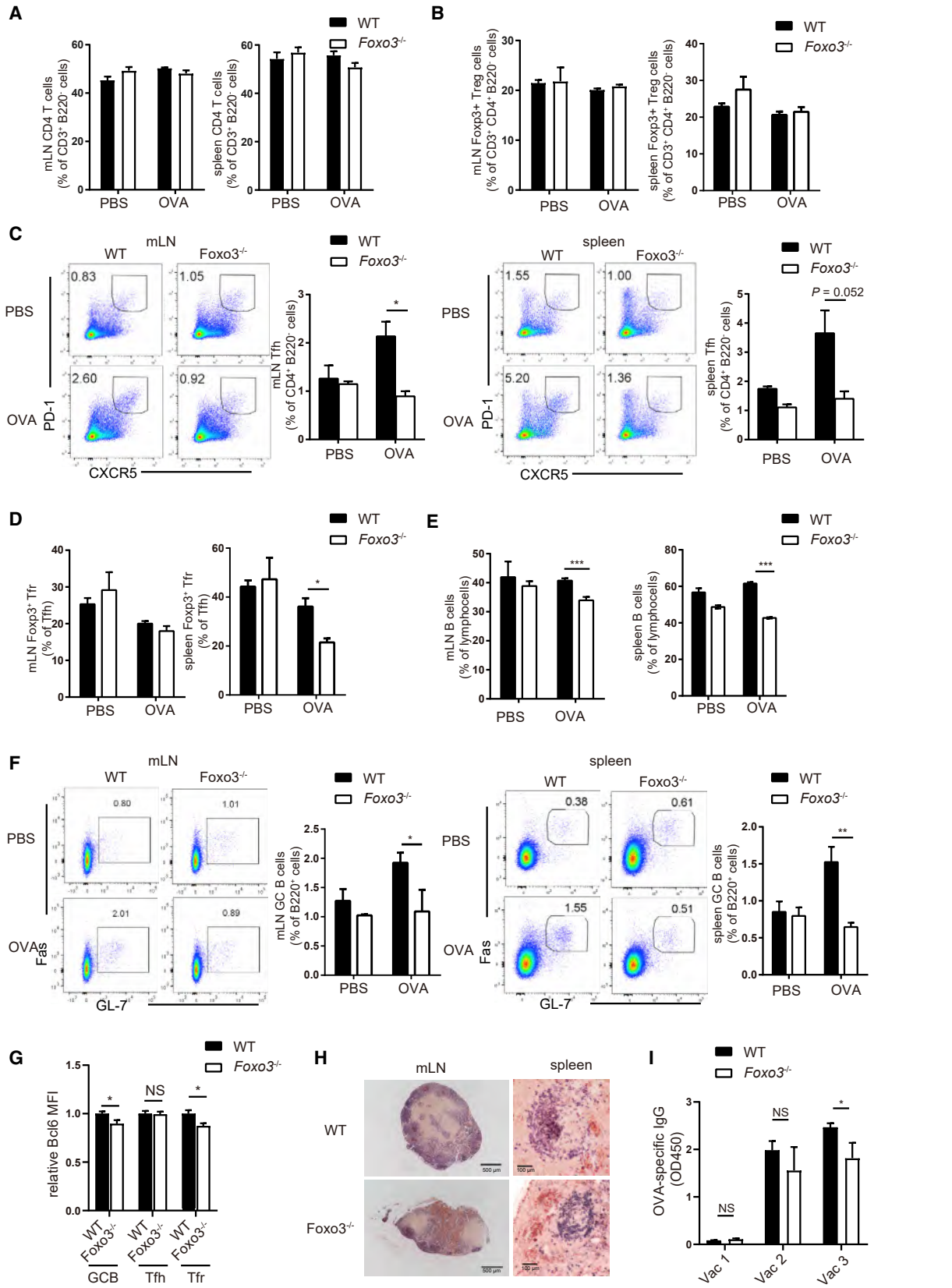
SUMMARY

Follicular helper T cells (Tfhs) are essential for germinal center (GC) B cell maturation and antibody development. However, the intrinsic mechanisms that regulate Tfh differentiation are largely unknown. Here, we demonstrate that the frequencies of Tfhs and GC B cells, as well as interleukin-21 (IL-21) and anti-ovalbumin (OVA) antibodies, are markedly decreased in forkhead box O3 (Foxo3) knockout mice immunized with OVA. Using mixed bone marrow chimeras and lymphocyte-repopulated *Rag1*$^{-/-}$ mice proves that wild-type (WT), but not Foxo3-deficient T cells provoke GC B cell maturation and antibody production. Deficiency of Foxo3 inhibits inducible T cell co-stimulator (ICOS)-induced Tfh differentiation. Chromatin immunoprecipitation assay results suggest that Foxo3 is able to bind to the IL-21 promoter and regulate IL-21 secretion. In conclusion, our study unveils a critical role of Foxo3 in the regulation of Tfh differentiation and IL-21 production. Modulating Foxo3 activity may be beneficial for enhancing or preventing antibody-mediated immune responses.

INTRODUCTION

Follicular helper T cells (Tfhs) are specialized providers of T cell help to B cells and are essential for germinal center (GC) formation and for the development of most high-affinity antibodies and memory B cells (Crotty, 2014; Song and Craft, 2019). Tfhs express a unique combination of effector molecules that are critical for their development and function, including high levels of the surface receptors inducible T cell co-stimulator (ICOS), CD40 ligand (CD40L), PD-1, the cytokine interleukin-21 (IL-21), and the transcription factors Bcl6 and c-Maf. These molecules play critical roles in promoting the activation, differentiation, and survival of Tfhs (Crotty, 2014; Song and Craft, 2019). Without signals from CD28, ICOS/ICOS-L, or CD40/CD40L, CD4$^+$ T cells fail to upregulate CXCR5, resulting in reduced numbers of Tfhs and impaired GC formation (Choi et al., 2011; Ma et al., 2012; Salek-Ardakani et al., 2011). Furthermore, Tfhs have also been shown to preferentially secrete the IL-21 cytokine, which is pivotal for GC formation, GC B cell proliferation, and B cell maturation (Crotty, 2011).

The forkhead box O (Foxo) family of transcription factors regulates diverse gene expression programs and affects many cellular processes (Eijkelenboom and Burgering, 2013). In mammals, the Foxo subfamily is comprised of four members, namely, Foxo1, Foxo3, Foxo4, and Foxo6 (Brown and Webb, 2018; Ohkura and Sakaguchi, 2010). Foxo1 and Foxo3 are the primary isoforms expressed in the immune system, which have been co-opted to regulate specialized characteristics of lymphocyte homeostasis (Deng et al., 2018; Hedrick et al., 2012; Luo and Li, 2018). Foxo1 is crucial for maintaining *Il7r* transcription. Compromised IL-7Rα expression results in blunted responses in Foxo1-deficient naive T cells to IL-7 survival signals *in vitro* and lymphopenia-triggered homeostatic proliferation *in vivo* (Kerdiles et al., 2009; Ouyang et al., 2009). T cells purified from *Foxo3*$^{-/-}$ mice showed no defect in proliferation or survival after *in vitro* stimulation (Dejean et al., 2009). Young mice with a conditional deletion of Foxo1 in CD4$^+$ T cells also exhibited a noticeable decrease in the proportion and number of Foxp3$^+$ regulatory T (Treg) cells (Kerdiles et al., 2010). In contrast to the dominant role of Foxo1 in controlling trafficking molecules and IL-7Rα expression in mature T cells, Foxo3 and Foxo1 cooperatively regulate Treg cell differentiation, as shown by diminished numbers of thymic and splenic Treg cells (Ouyang et al., 2010).



(legend on next page)

Foxo1 has also been reported to regulate a Tfh gene program exemplified by the negative regulation of Bcl6 (Stone et al., 2015). Mice with a T-cell-specific deletion of *Foxo1* spontaneously accumulate a large number of CXCR5+PD1+ Tfhs, and this corresponds with the appearance of GCs, class-switched B cells, and DNA-specific antibodies (Kerdiles et al., 2010). However, whether Foxo3 is involved in Tfh differentiation and function remains obscure.

In this study, we showed that Foxo3 is critical for humoral immunity by maintaining Tfh function, GC formation, and antibody production. We also verified that Foxo3 regulates Tfh function by binding to the *Il21* promoter, enhancing IL-21 expression.

RESULTS

Foxo3 Deficiency Impairs the Frequency and Function of Tfhs

Genotype identification of *Foxo3* and *Foxo1* is shown in Figure S1A. *Foxo1* mRNA expression was not significantly different between splenocytes from *Foxo3*$^{-/-}$ mice and WT littermates (Figure S1B). Moreover, no significant difference of *Foxo1* mRNA expression was observed between *Foxo3*$^{-/-}$ CD4 T cells and WT CD4 T cells after anti-CD3/CD28 stimulation (Figure S1C). To study the role of Foxo3 in Tfhs, we immunized *Foxo3*$^{-/-}$ mice and their WT littermates with ovalbumin (OVA) and then analyzed CD4$^+$ T cells subtypes from mesenteric lymph nodes (mLNs) and spleen. Compared to WT groups, the frequency of CD4$^+$ T cells and Foxp3$^+$ Treg cells in spleen or lymph nodes exhibited no obvious difference (Figures 1A and 1B). However, the frequency of mLN Tfhs in the CD4$^+$B220$^-$ cells was significantly decreased in *Foxo3*$^{-/-}$ mice after OVA immunization ($p < 0.05$; Figure 1C). The frequency of spleen Tfhs in CD4$^+$B220$^-$ cells was also markedly reduced in *Foxo3*$^{-/-}$ mice compared to WT mice (Figure 1C). Interestingly, the frequency of spleen Foxp3$^+$ T follicular regulatory (Tfr) cells in Tfhs was also obviously decreased in *Foxo3*$^{-/-}$ mice with OVA immunization, although the frequencies of mLN Tfr cells were not significantly different (Figure 1D). Tfhs mediate the differentiation of GC B cells into memory and plasma cells, so we analyzed GC B cells and plasma cells. Our results showed that the frequency of both mLNs and spleen B220$^+$ B cells were significantly decreased in *Foxo3*$^{-/-}$ mice after OVA stimulation (Figure 1E). As a symbol of B cell activation and antibody production, GC B cells (B220$^+$ GL-7$^+$ Fas$^+$) were also significantly reduced in the mLNs and spleen of *Foxo3*$^{-/-}$ mice ($p < 0.05$; Figure 1F). Compared with the WT counterparts, Bcl6 expression was declined in *Foxo3*$^{-/-}$ GC B and Tfr cells ($p < 0.05$) but not in Tfhs; thus, Bcl6 expression in Tfhs was not affected by the deficiency of Foxo3 (Figure 1G). Immunohistochemistry results showed that GC size was smaller in OVA-vaccinated *Foxo3*$^{-/-}$ mice than in WT mice (Figure 1H). Key markers, including B220 and Bcl6, were expressed in GCs. Serum levels of OVA-specific immunoglobulin G (IgG) were lower in *Foxo3*$^{-/-}$ mice than in WT mice after the third immunization ($p < 0.01$; Figure 1I).

Loss of Foxo3 Reduced the Conversion and Function of Tfhs *In Vitro*

To investigate the influence of Foxo3 on Tfh polarization, we monitored the conversion of naive CD4$^+$CD25$^-$ T cells isolated from WT and *Foxo3*$^{-/-}$ mice into CD4$^+$CXCR5$^+$PD-1$^+$ Tfhs *in vitro*. We found that the conversion ratio of Tfhs was markedly decreased in the *Foxo3*$^{-/-}$ group (Figure 2A). Furthermore, we analyzed cytokines produced by Tfhs, including IL-4 and IL-21. *Foxo3*$^{-/-}$ Tfhs secreted relatively lower levels of IL-4 (Figure 2B), although this trend did not reach significance. However, IL-21, a key cytokine secreted by Tfhs, was markedly decreased in *Foxo3*$^{-/-}$ Tfhs compared to WT Tfhs (Figure 2C). Accordingly, the levels of IL-21 in the supernatant were also significantly decreased in the *Foxo3*$^{-/-}$ CD4$^+$ T cell culture system (Figure 2D). Nevertheless, mRNA expression levels of *Il4* and *Il21* in *Foxo3*$^{-/-}$ Tfhs declined remarkably (Figure 2E). In addition to cell-surface-expressed molecules and cytokines, mRNA expression of Tfh-related transcription factors, including *Bcl6*, *Cmaf,* and *Batf*, was similarly decreased (Figure 2E).

The selection of affinity-matured antibody-producing B cells is supported by interactions with Tfhs. To determine whether a loss of Foxo3 signaling attenuates this Tfh function, we co-cultured *Foxo3*$^{-/-}$ or WT B cells and *Foxo3*$^{-/-}$ or WT Tfhs *in vitro* for 6 days. Compared with WT B + WT Tfh coculture group, the percentage of GC B cells declined in both the WT B + *Foxo3*$^{-/-}$ Tfh coculture group and *Foxo3*$^{-/-}$ B + WT Tfh coculture group. In the GC B-cell-generating system, a loss of Foxo3 reduced the ability of Tfhs to induce GC B cells and plasmablast cells and, meanwhile, impaired the differentiation and function of B cells (Figure 2F).

Figure 1. Loss of Foxo3 Decreases Tfhs and Humoral Immunity

WT and *Foxo3*$^{-/-}$ mice were immunized with OVA, and T cell subsets of mLNs and spleens were determined by flow cytometry.

(A) The percentage of CD3$^+$ CD4$^+$ B220$^-$ T cells in spleens and mLNs was detected by flow cytometry.

(B) The percentage of Foxp3$^+$ CD3$^+$ CD4$^+$ B220$^-$ Treg cells in spleens and mLNs after OVA immunization was detected by flow cytometry.

(C) The percentage of CXCR5$^+$ PD-1$^+$ CD4$^+$ B220$^-$ Tfhs in the *Foxo3*$^{-/-}$ group and WT group in mLNs and spleens was detected by flow cytometry.

(D) The percentage of Foxp3$^+$ CXCR5$^+$ PD-1$^+$ CD4$^+$ B220$^-$ Tfr cells in mLNs and spleens was detected by flow cytometry.

WT and *Foxo3*$^{-/-}$ mice were immunized with OVA, and B cell subsets of mLN and spleen were determined by flow cytometry.

(E) The percentage of B220$^+$ CD3$^-$ B cells in mLNs and spleens was detected by flow cytometry.

(F) The percentage of B220$^+$ GL-7$^+$Fas$^+$ GC B cells in mLNs and spleens was detected by flow cytometry.

(G) MFI of Bcl6 on splenic GC B cells, Tfr cells, and Tfhs.

(H) Immunohistochemical staining of mLN and spleen sections from C57BL/6 and *Foxo3*$^{-/-}$ mice 7 days after immunization. Blue is B220, identifying B cell follicles, and red is Bcl6, revealing the location of GC B cells. Scale bar, 500 μm and 100 μm.

(I) After 7 days of each OVA immunization, levels of serum OVA-specific IgG were detected by ELISA.

Data are displayed as mean ± SEM of n = 4–6 independent experiments. Significance was determined by one-way analysis of variance. *$p < 0.05$, **$p < 0.01$. See also Figure S1.

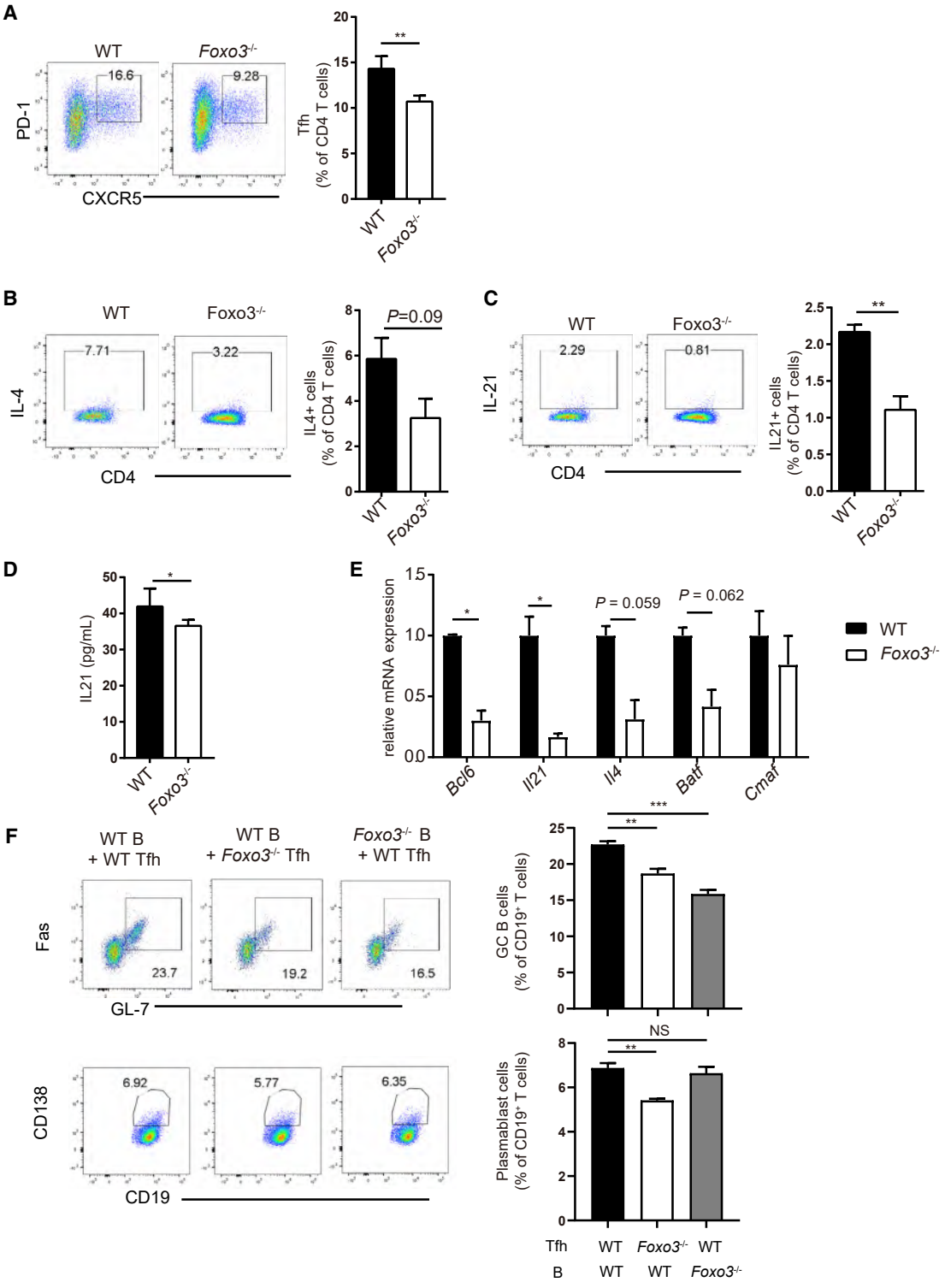


Figure 2. Foxo3 Deficiency Reduced the Conversion and Function of Tfhs *In Vitro*

(A) The conversion ratios of Tfhs in response to Tfh polarization treatment for 6 days *in vitro* were detected by flow cytometry.

(B and C) IL-4 (B) and IL-21 (C) secretions of $CD4^+$ T cells were detected by flow cytometry.

(D) Levels of IL-21 in the supernatant were detected by ELISA.

(legend continued on next page)

Loss of Foxo3 Impaired the Differentiation and Function of Tfhs *In Vivo*

To assess whether Foxo3 deficiency impairs the differentiation and function of Tfhs *in vivo*, we adoptively transferred 50%: 50% (*Foxo3*$^{-/-}$ CD45.2^{+}: WT CD45.1^{+} versus WT CD45.2^{+}: WT CD45.1^{+}) mixed bone marrow cells into irradiated CD45.1^{+} recipient mice separately (2 × 10^{7} cells/mouse). Six weeks later, the recipient mice were immunized with OVA for 3 times (day 1, day 14, and day 28), and the differentiation and function of Tfhs were analyzed.

As expected, the frequencies of CD45.2^{+} Tfhs in spleen and mLNs were reduced significantly in mice with mixed bone marrow chimera from CD45.2^{+} *Foxo3*$^{-/-}$ and CD45.1^{+} WT mice (Figure 3A). IL-21 secretions by CD4^{+} T cells and Tfhs were significantly declined in mLNs from mice with *Foxo3*$^{-/-}$ mixed bone marrow chimera (Figure 3B). Furthermore, the percentage of splenic and mLN GC B cells markedly decreased in Foxo3-deficient mixed bone marrow chimeric mice compared with mice reconstituted with WT bone marrow (Figure 3C). Similarly, lower percentages of splenic and mLN plasma cells were observed in chimeric Foxo3-deficient mice (Figure 3D). The development of Foxp3^{+}CD4^{+} T cells in spleens and mLNs showed no significant difference between groups (Figure 3E). Foxo3-deficient mixed bone marrow chimeric mice also showed significantly less OVA-specific IgG than WT mice (Figure 3F). Furthermore, we also compared Tfhs in the same bone marrow chimeric mice. We found similar changes of CD45.2^{+} *Foxo3*$^{-/-}$ Tfhs and CD45.1^{+} WT Tfhs in the same recipients (Figures S2A–S2E).

To exclude the intrinsic effect of Foxo3 on GC B cells, we adoptively transferred WT B cells and Foxo3-deficient naive CD4^{+}CD25^{-} T cells into Rag1$^{-/-}$ mice. Rag1$^{-/-}$ mice received WT naive CD4^{+}CD25^{-} T cells, and WT B cells served as a control. Spleens and mLNs from recipient mice were isolated and analyzed after 3 doses of OVA immunization. The results unveiled that the percentages of Tfhs markedly decreased in both spleens and mLNs in *Rag1*$^{-/-}$ mice that received Foxo3-deficient CD4^{+} CD25^{-} T cells and WT B cells (Figure 3G). Accordingly, the frequency of IL-21^{+}CD4^{+} T cells and IL-4^{+}CD4^{+} T cells both decreased in the spleens of *Rag1*$^{-/-}$ mice (Figure 3H). Furthermore, the percentage of GC B cells (Figure 3I) and IgG^{+} plasma cells (Figure 3J) markedly decreased in *Rag1*$^{-/-}$ mice co-transferred with Foxo3-deficient CD4^{+}CD25^{-} T cells and WT B cells compared to *Rag1*$^{-/-}$ mice that received WT CD4^{+}CD25^{-} T cells and WT B cells. However, Foxp3^{+}Treg cell development in both spleens and mLNs showed no obvious change by deficiency of Foxo3 (Figure 3K). Correspondingly, less levels of titration serum OVA-specific IgG (Figure 3L) were observed in *Rag1*$^{-/-}$ mice reconstituted with Foxo3-deficient CD4^{+}CD25^{-} T cells and WT B cells.

During GC B cells isotype switching, the percentage of IgM^{-}IgD^{-} GC B cells was unchanged (Figure S3A). In this study, we also found that the percentage of CXCR4^{-}CD86^{+} GC B cells (light zone) but not CXCR4^{+}CD86^{-} GC B cells (dark zone) decrease in Foxo3-deficient mice (Figure S3B). Foxo3 deficiency mainly affects the light zone of GC, where Tfhs provide help signals for GC B cell maturation after OVA immunization. Taken together, our findings indicate that Foxo3 indeed plays an essential role in the humoral immune response by affecting the differentiation and function of Tfhs and inducing GC B cells into plasma cells.

Foxo3 Deficiency Disrupts the Stimulation of ICOS to Tfhs

ICOS is a potent co-receptor distinct from CD28 that is induced on activated T cells and highly expressed on Tfhs. ICOS signaling is necessary for complete GC development, T-cell-dependent B cell help, and antibody class switching due to a role for ICOS in the differentiation of activated T cells to Tfhs (Crotty, 2014). In this study, when CD4^{+} T cells were stimulated with anti-CD3 and anti-ICOS antibodies, along with increased Tfh differentiation, we observed upregulated *Foxo3* mRNA expression (Figure 4A). We then investigated whether Foxo3 affects ICOS-promoted Tfh differentiation. Results revealed that ICOS stimulation promotes CXCR5^{+}PD1^{+}Tfhs in WT CD4^{+} T cells but not in Foxo3-deficient CD4^{+} T cells (Figure 4B). Furthermore, CD69, a marker of active Tfhs, was not obviously distinct from *Foxo3*$^{-/-}$ or WT Tfhs (Figure 4C). The proportion of apoptotic cells markedly decreased in Foxo3-deficient Tfhs compared to WT Tfhs (Figure 4D). Meanwhile, Tfh-related *IL-21* mRNA expression was also upregulated in WT CD4^{+} T cells but not in *Foxo3*$^{-/-}$ CD4^{+} T cells in response to ICOS stimulation (Figure 4E). These findings indicate that Foxo3 regulation is necessary for ICOS-induced Tfh differentiation.

Foxo3 Directly Binds to the IL-21 Promoter

To investigate the intrinsic mechanism of Foxo3's regulation on IL-21 secretion from Tfhs, the upstream factors regulating IL-21 were investigated. The DNA binding sequence of Foxo3 was predicted using the JASPAR website (Figure 4F). In the IL-21 promoter region (−865/+288 region), potential transcription factor binding sites are shown in Figure 4G based on bioinformatics predictions. To further verify Foxo3-binding sites to the IL-21 core promoter region, we performed a chromatin immunoprecipitation (ChIP) assay. As shown in Figure 4H, after immunoprecipitation with anti-Foxo3a antibodies, a positive band of approximately −529 to −382 bp appeared in the bound DNA of WT Tfhs; however, no amplification of bound DNA from Foxo3-deficient Tfhs was present. Precipitation with an anti-Foxo3a antibody did not show a positive DNA band in −380 to −126 bp and −181 to −102 bp, and a significant difference of a positive DNA band between WT and *Foxo3*$^{-/-}$ Tfh was revealed in −529 to −382 bp (Figures 4H and 4I).

DISCUSSION

Foxo proteins are involved in the differentiation of diverse effector T cell subsets. Deletion of Foxo1 results in a significant

(E) RNA expressions of Tfh transcription factors and cytokines were detected by real-time PCR.

(F) In a co-culture system, the proportions of GL-7^{+} Fas^{+} GC B cells and CD19^{+} CD138^{+} plasmablast cells induced by Tfhs were detected by flow cytometry. Data are displayed as mean ± SEM of n = 4–6 independent experiments. Significance was determined by Student's t test and one-way analysis of variance. *p < 0.05, **p < 0.01, ***p < 0.001.

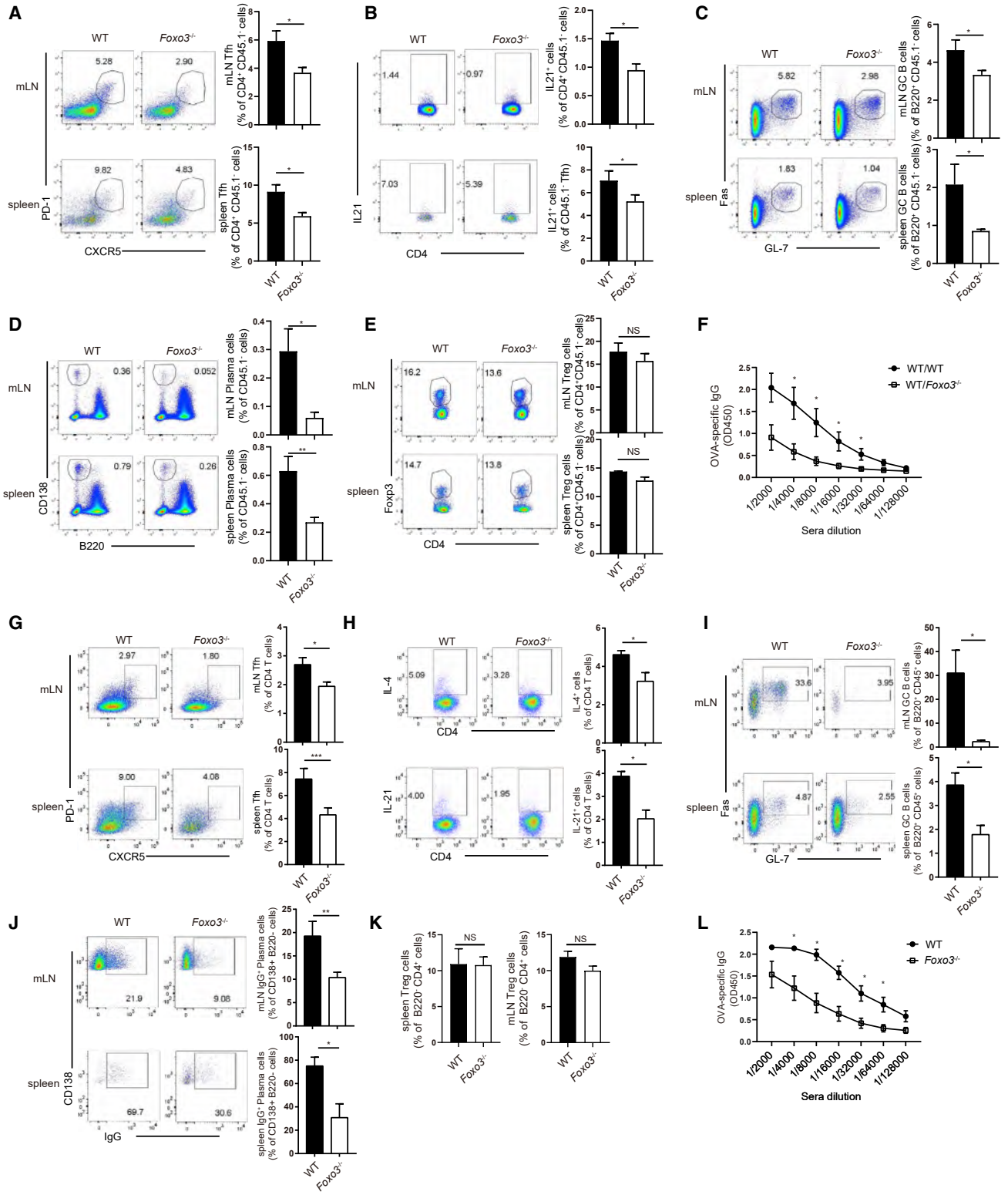


Figure 3. Loss of Foxo3 Impaired the Intrinsic Ability of Tfh Inducing GCBs into Plasma Cells *In Vivo*

Bone marrow cells from WT or *Foxo3*$^{-/-}$ (CD45.2^{+}) mice were mixed with cells from competitor (CD45.1^{+}) mice at a 1:1 ratio and transferred to the mixed cells, totaling 2 × 10^{7} cells, into lethally irradiated congenic (CD45.1^{+}) mice (n = 6 mice/group). After reconstitution, the recipient mice were immunized with OVA for 3 times. Percentages of spleen and mLN cells were determined by flow cytometry.

(legend continued on next page)

increase in the generation of Tbet$^+$IFN-γ^+Th1 and RORγt$^+$IL-17$^+$Th17 cells (Lainé et al., 2015; Ouyang et al., 2009, 2010). Compared to their WT counterparts, CD4$^+$ T cells from Foxo3-deficient mice produce higher levels of Th1 cytokines, such as IL-2 and IFN-γ, and exhibit enhanced expression of Th2 cytokines, such as IL-4, IL-5, IL-6, and IL-10, in response to Th1- or Th2-polarizing conditions (Lin et al., 2004). Foxo1 and Foxo3 have been shown to cooperatively regulate the differentiation of Foxp3$^+$ Treg cells as well (Ouyang et al., 2010; Wang et al., 2014). Deletion of Foxo1 promotes CD8$^+$ T cell effector functions (Rao et al., 2012). Deletion of Foxo3 enhances the accumulation of primary-antigen-specific CD8$^+$ T cells primarily by inhibiting their apoptosis, while their rate of cell division remains unaffected (Togher et al., 2015).

Foxo transcription factors have also been reported to regulate the differentiation of CD4+ T cells into Tfhs. ICOS signaling inactivates the transcription factor Foxo1. Foxo1 regulates a Tfh gene program exemplified by negative regulation of Bcl6, and forced nuclear localization of Foxo1 prevents Tfh differentiation (Stone et al., 2015). Mice with T-cell-specific Foxo1 deletion accumulate a large population of Tfhs and develop B cell autoimmunity, which is manifested by GC formation and production of circulating anti-DNA antibodies (Kerdiles et al., 2010).

In naive *Foxo3*$^{-/-}$ mice, we did not see significant changes in CD4$^+$ T cell and Tfh frequencies compared to their WT littermates. However, after OVA immunization, we observed decreased CXCR5$^+$PD-1$^+$Tfhs in spleens and mLNs of *Foxo3*$^{-/-}$ mice, as well as decreased GC B and plasma cells. Accordingly, levels of OVA-specific IgG markedly declined. These results indicate that Foxo3 deficiency attenuates the differentiation and function of Tfhs and humoral immunity. Unlike inactivation of Foxo1 by ICOS signaling (Stone et al., 2015), we found that ICOS stimulation upregulated Foxo3 expression of CD4$^+$ T cells and enhanced Tfh differentiation. This enhanced Tfh differentiation was neutralized when Foxo3 was deficient, indicating that Foxo3 is distinct from Foxo1 and is necessary in ICOS-induced Tfh differentiation.

One of the characteristic features of Foxo transcription factors is their control of the cell cycle and apoptosis. Previous studies have shown that the *Foxo3* gene promotes apoptosis of B cells, T cells, hematopoietic stem cells, and tumor cells (Ottens et al., 2018; Wang et al., 2014). Foxo transcription factors promote cell growth inhibitory and/or apoptosis signaling by either inducing the expression of multiple pro-apoptotic members of the Bcl2-family of mitochondria-targeting proteins, stimulating expression of death receptor ligands, such as Fas ligand and tumor-necrosis-factor-related apoptosis-inducing ligand (TRAIL), or by enhancing levels of various cyclin-dependent kinase inhibitors (CDKIs) (Zhang et al., 2011). In both acute and chronic viral infection models, the loss of Foxo3 results in an enhanced accumulation of antigen-specific T cells, which is associated with attenuated cell death (Sullivan et al., 2012a, 2012b). In this study, we also observed reduced rates of apoptosis of Tfhs in *Foxo3*$^{-/-}$ mice; however, the prevalence of apoptosis of Tfhs with or without ICOS stimulation was not different, suggesting that decreased Tfh differentiation is not apoptosis related.

IL-21 is essential for Tfh development (Nurieva et al., 2008; Vogelzang et al., 2008). Tfhs are essential for the formation and maintenance of GCs, and their differentiation relies on the transcriptional regulators B cell lymphoma 6 (Bcl6) and STAT3, which are also mobilized by IL-21 (Crotty, 2014). In this study, using ChIP assays, we revealed that Foxo3 binds to the IL-21 promoter of CD4$^+$ T cells and enhances the expression of IL-21, which may result in upregulated Tfh differentiation, GC formation, and antibody production. These findings imply that Foxo1 and Foxo3 regulate distinct aspects of Tfh differentiation. Foxo1 primarily regulates a Tfh gene program exemplified by negative regulation of Bcl6, while Foxo3 promotes Tfh generation by enhancing IL-21 production.

There are limitations in the current study. First, decreased Tfhs was observed in OVA-challenged *Foxo3*$^{-/-}$ and *Foxo3*$^{-/-}$/WT mixed bone marrow chimeric mice. Although we have revealed that Foxo3 regulation is necessary for ICOS-induced Tfh differentiation, the Tfh defect might also be due to the insufficient ICOS signaling. Thus, the possibility that Foxo3 regulates ICOS expression needs to be addressed in the future. Second, in *Foxo3*$^{-/-}$ mice or mixed bone marrow chimeric mice, we also observed markedly decreased GC B cells and OVA-specific antibodies after OVA immunization. Although Foxo3-deficient Tfhs account for the impaired B cell differentiation and function, Foxo3 might also have direct impact on B cells or have impact on other T-cell-dependent signals that are necessary for B cell maturation, such as CD40L. Further studies of Foxo3 on B cell differentiation and function are needed.

In summary, during Tfh differentiation, Foxo3 is significantly upregulated in CD4$^+$ T cells. The frequency of Tfh and GC B cells,

(A) Percentages of CD45.1$^-$CXCR5$^+$ PD-1$^+$ CD4$^+$ B220$^-$ Tfhs in spleens and mLNs.
(B) Percentages of IL-21$^+$ CD45.1$^-$ CD4$^+$ B220$^-$ T cells and IL-21$^+$ CD45.1$^-$ Tfhs in mLNs.
(C and D). Percentages of CD45.1$^-$ B220$^+$ GL-7$^+$ Fas$^+$ GC B cells (C) and B220$^-$CD138$^+$ CD45.1$^-$ plasma cells (D) in spleens and mLNs were detected by flow cytometry.
(E) Percentages of CD45.1$^-$ Foxp3$^+$ CD4$^+$ Treg cells in spleen and lymph nodes were detected by flow cytometry.
(F) Levels of OVA-specific IgG antibody were detected by ELISA.
CD4$^+$CD25$^-$ T cells (from WT or *Foxo3*$^{-/-}$ mice) and B cells (from WT mice) were adoptively transferred into *Rag1*$^{-/-}$ mice and immunized with OVA (three times at day 1, day 14, day 28), and spleens and mLNs were analyzed 5 days post-immunization.
(G) The percentage of CXCR5$^+$ PD-1$^+$ CD4$^+$ B220$^-$ Tfhs in spleens and mLNs was detected by flow cytometry.
(H) IL-4 and IL-21 secretion from CD4 T cells in spleens was detected by flow cytometry.
(I and J) The percentage of CD45$^+$ B220$^+$ GL-7$^+$ Fas$^+$ GC B cells (I) and B220$^-$CD138$^+$ IgG$^+$ plasma cells (J) in spleens and mLNs was detected by flow cytometry.
(K) The percentage of Foxp3$^+$ Treg cells in spleens and mLNs.
(L) Titration serum OVA-specific IgG antibody was detected by ELISA.
Data are displayed as mean ± SEM of n = 4–6 independent experiments. Significance was determined by one-way analysis of variance. *p < 0.05, **p < 0.01, ***p < 0.001. See also Figures S2 and S3.

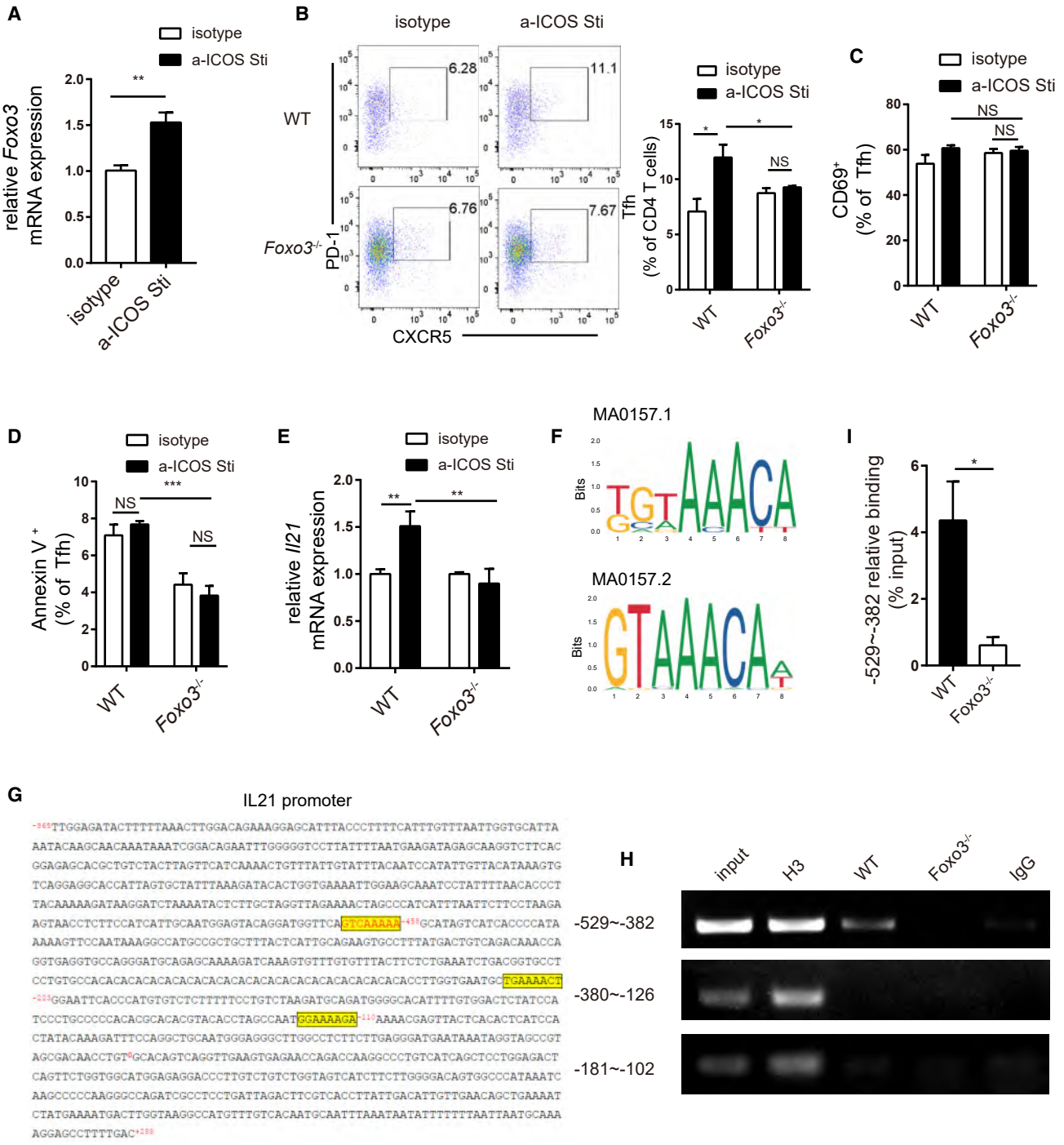


Figure 4. Foxo3 Deficiency Disrupts the Stimulation of ICOS to Tfh, and Identification of the Core Promoter of Foxo3 Gene Regulation

CD4 T cells from *Foxo3*$^{-/-}$ mice and WT mice were stimulated with a-CD3 and a-CD28 antibodies for 3 days. Anti-ICOS antibody (2 μg/ml) was added during Tfh polarization treatment.

(A) *Foxo3* mRNA expression was detected by real-time PCR.

(B) CXCR5^{+} PD1^{+} CD4^{+} Tfhs were detected by flow cytometry.

(C and D) The proportion of active CD69^{+} Tfhs (C) and the apoptosis of Tfhs (D) were detected by flow cytometry.

(E) mRNA expression of *IL-21* was detected by real-time PCR.

(F) DNA binding sequences of Foxo3 were predicted by the JASPAR website.

(G) Nucleotide sequence of the IL-21 promoter region. Potential regulatory elements identified based on the Transcription Factor Binding Sites database TRANSFAC are shown underlined and identified by the appropriate symbols.

(legend continued on next page)

as well as the production of IL-21- and OVA-specific antibodies, were markedly decreased in *Foxo3*$^{-/-}$ mice under OVA immunization. WT T cells, but not Foxo3-deficient T cells, provoked WT GC B cell and plasma cell maturation and antibody production. The deficiency of Foxo3 and ICOS stimulation failed to promote Tfh differentiation and IL-21 production. The ChIP assay results strongly suggested that Foxo3 binds to the IL-21 promoter and is involved in the regulation of IL-21 secretion of CD4^{+} T cells.

In conclusion, our study unveiled a critical role for Foxo3 in the regulation of Tfh differentiation and function. Modulating the activity of Foxo3 may prove beneficial in immunotherapeutic strategies for enhancing or preventing antibody-mediated immune responses.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead Contact
  - Materials Availability
  - Data and Code Availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
- METHOD DETAILS
  - Cell isolation
  - Flow cytometry
  - Generation of Tfh *in vitro*
  - Adoptive transfer experiments
  - Bone marrow chimera
  - Quantitative real-time PCR
  - Measurement of OVA-specific IgG and IL-21
  - Immunohistochemistry
  - Chromatin immunoprecipitation assay
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2020.107621.

ACKNOWLEDGMENTS

This study was supported by grants from the National Natural Science Foundation of China (81601364, 81601388, 81372737, and 81870399).

AUTHOR CONTRIBUTIONS

All listed authors participated meaningfully in the study, and they have seen and approved the submission of this manuscript. H.Q. and D.T. participated in performing the research, analyzing the data, and initiating the original draft of the article. M.L., C.Z., H.J., L.L., X.Z., L.M., W.Z., and Q.G. participated in performing the research and collecting the data. D.Z. and D.T. established the hypotheses, supervised the studies, analyzed the data, and co-wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: July 8, 2019
Revised: January 27, 2020
Accepted: April 16, 2020
Published: May 12, 2020

REFERENCES

Brown, A.K., and Webb, A.E. (2018). Regulation of FOXO Factors in Mammalian Cells. Curr. Top. Dev. Biol. *127*, 165–192.

Choi, Y.S., Kageyama, R., Eto, D., Escobar, T.C., Johnston, R.J., Monticelli, L., Lao, C., and Crotty, S. (2011). ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. Immunity *34*, 932–946.

Crotty, S. (2011). Follicular helper CD4 T cells (TFH). Annu. Rev. Immunol. *29*, 621–663.

Crotty, S. (2014). T follicular helper cell differentiation, function, and roles in disease. Immunity *41*, 529–542.

Dejean, A.S., Beisner, D.R., Ch'en, I.L., Kerdiles, Y.M., Babour, A., Arden, K.C., Castrillon, D.H., DePinho, R.A., and Hedrick, S.M. (2009). Transcription factor Foxo3 controls the magnitude of T cell immune responses by modulating the function of dendritic cells. Nat. Immunol. *10*, 504–513.

Deng, Y., Wang, F., Hughes, T., and Yu, J. (2018). FOXOs in cancer immunity: Knowns and unknowns. Semin. Cancer Biol. *50*, 53–64.

Eijkelenboom, A., and Burgering, B.M. (2013). FOXOs: signalling integrators for homeostasis maintenance. Nat. Rev. Mol. Cell Biol. *14*, 83–97.

Hedrick, S.M., Hess Michelini, R., Doedens, A.L., Goldrath, A.W., and Stone, E.L. (2012). FOXO transcription factors throughout T cell biology. Nat. Rev. Immunol. *12*, 649–661.

Kerdiles, Y.M., Beisner, D.R., Tinoco, R., Dejean, A.S., Castrillon, D.H., DePinho, R.A., and Hedrick, S.M. (2009). Foxo1 links homing and survival of naive T cells by regulating L-selectin, CCR7 and interleukin 7 receptor. Nat. Immunol. *10*, 176–184.

Kerdiles, Y.M., Stone, E.L., Beisner, D.R., McGargill, M.A., Ch'en, I.L., Stockmann, C., Katayama, C.D., and Hedrick, S.M. (2010). Foxo transcription factors control regulatory T cell development and function. Immunity *33*, 890–904.

Lainé, A., Martin, B., Luka, M., Mir, L., Auffray, C., Lucas, B., Bismuth, G., and Charvet, C. (2015). Foxo1 Is a T Cell-Intrinsic Inhibitor of the RORγt-Th17 Program. J. Immunol. *195*, 1791–1803.

Lin, L., Hron, J.D., and Peng, S.L. (2004). Regulation of NF-kappaB, Th activation, and autoinflammation by the forkhead transcription factor Foxo3a. Immunity *21*, 203–213.

Luo, C.T., and Li, M.O. (2018). Foxo transcription factors in T cell biology and tumor immunity. Semin. Cancer Biol. *50*, 13–20.

Ma, C.S., Deenick, E.K., Batten, M., and Tangye, S.G. (2012). The origins, function, and regulation of T follicular helper cells. J. Exp. Med. *209*, 1241–1253.

Nurieva, R.I., Chung, Y., Hwang, D., Yang, X.O., Kang, H.S., Ma, L., Wang, Y.H., Watowich, S.S., Jetten, A.M., Tian, Q., and Dong, C. (2008). Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. Immunity *29*, 138–149.

Ohkura, N., and Sakaguchi, S. (2010). Foxo1 and Foxo3 help Foxp3. Immunity *33*, 835–837.

Ottens, K., Hinman, R.M., Barrios, E., Skaug, B., Davis, L.S., Li, Q.Z., Castrillon, D.H., and Satterthwaite, A.B. (2018). Foxo3 Promotes Apoptosis of B Cell

(H and I) Binding of the specific fragment (H) and relative binding levels of the −259 to −382 fragment (I) in the IL-21 core promoter was detected by ChIP and real-time PCR.

Data are displayed as mean ± SEM of n = 4–6 independent experiments. Significance was determined by Student's t test and one-way analysis of variance. *p < 0.05. **p < 0.01, ***p < 0.001.

Receptor-Stimulated Immature B Cells, Thus Limiting the Window for Receptor Editing. J. Immunol. *201*, 940–949.

Ouyang, W., Beckett, O., Flavell, R.A., and Li, M.O. (2009). An essential role of the Forkhead-box transcription factor Foxo1 in control of T cell homeostasis and tolerance. Immunity *30*, 358–371.

Ouyang, W., Beckett, O., Ma, Q., Paik, J.H., DePinho, R.A., and Li, M.O. (2010). Foxo proteins cooperatively control the differentiation of Foxp3+ regulatory T cells. Nat. Immunol. *11*, 618–627.

Rao, R.R., Li, Q., Gubbels Bupp, M.R., and Shrikant, P.A. (2012). Transcription factor Foxo1 represses T-bet-mediated effector functions and promotes memory CD8(+) T cell differentiation. Immunity *36*, 374–387.

Rumble, J., and Segal, B.M. (2014). In vitro polarization of T-helper cells. Methods Mol. Biol. *1193*, 105–113.

Salek-Ardakani, S., Choi, Y.S., Rafii-El-Idrissi Benhnia, M., Flynn, R., Arens, R., Shoenberger, S., Crotty, S., Croft, M., and Salek-Ardakani, S. (2011). B cell-specific expression of B7-2 is required for follicular Th cell function in response to vaccinia virus. J. Immunol. *186*, 5294–5303.

Song, W., and Craft, J. (2019). T follicular helper cell heterogeneity: Time, space, and function. Immunol. Rev. *288*, 85–96.

Stone, E.L., Pepper, M., Katayama, C.D., Kerdiles, Y.M., Lai, C.Y., Emslie, E., Lin, Y.C., Yang, E., Goldrath, A.W., Li, M.O., et al. (2015). ICOS coreceptor signaling inactivates the transcription factor FOXO1 to promote Tfh cell differentiation. Immunity *42*, 239–251.

Sullivan, J.A., Kim, E.H., Plisch, E.H., Peng, S.L., and Suresh, M. (2012a). FOXO3 regulates CD8 T cell memory by T cell-intrinsic mechanisms. PLoS Pathog. *8*, e1002533.

Sullivan, J.A., Kim, E.H., Plisch, E.H., and Suresh, M. (2012b). FOXO3 regulates the CD8 T cell response to a chronic viral infection. J. Virol. *86*, 9025–9034.

Togher, S., Larange, A., Schoenberger, S.P., and Feau, S. (2015). FoxO3 is a negative regulator of primary CD8+ T-cell expansion but not of memory formation. Immunol. Cell Biol. *93*, 120–125.

Vogelzang, A., McGuire, H.M., Yu, D., Sprent, J., Mackay, C.R., and King, C. (2008). A fundamental role for interleukin-21 in the generation of T follicular helper cells. Immunity *29*, 127–137.

Wang, Y., Zhou, Y., and Graves, D.T. (2014). FOXO transcription factors: their clinical significance and regulation. BioMed Res. Int. *2014*, 925350.

Zhang, X., Tang, N., Hadden, T.J., and Rishi, A.K. (2011). Akt, FoxO and regulation of apoptosis. Biochim. Biophys. Acta *1813*, 1978–1986.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rat monoclonal anti-CD4 Pacific Blue (clone GK1.5)	Biolegend	Cat#100428; RRID: AB_493647
Rat monoclonal anti-B220 FITC (clone RA3-6B2)	Biolegend	Cat#103206; RRID: AB_312991
Rat monoclonal anti-CD249 (PD-1) PE (clone RMP1-30)	eBioscience	Cat#12-9981-83; RRID: AB_466291
Rat monoclonal anti-CD249 (PD-1) PE/Cy7 (clone RMP1-30)	Biolegend	Cat#109110; RRID: AB_572017
Armenian Hamster monoclonal anti-CD69 PE/Cy7 (clone H1.2F3)	Biolegend	Cat#104512; RRID: AB_493564
Rat monoclonal anti-CD45 PE/Cy7 (clone 30-F11)	Biolegend	Cat#103114; RRID: AB_312979
Rat monoclonal anti-CD19 FITC (clone 6D5)	Biolegend	Cat#115506; RRID: AB_313641
Rat monoclonal anti-GL-7 PE (clone GL7)	Biolegend	Cat#144608; RRID: AB_2562926
Rat monoclonal anti-GL-7 PerCP/Cy5.5 (clone GL7)	Biolegend	Cat#144610; RRID: AB_2562979
Mouse monoclonal anti-CD95 (Fas) APC (clone SA367H8)	Biolegend	Cat#152604; RRID: AB_2632899
Rat monoclonal anti-IgG1 APC (clone A85-1)	BD Biosciences	Cat#560089; RRID: AB_1645625
Rat monoclonal anti-CD138 Brilliant Violet 421 (clone 281-2)	Biolegend	Cat#142507; RRID: AB_11204257
Rat monoclonal anti-CD45.1 PE/Cy7 (clone A20)	Biolegend	Cat#110730; RRID: AB_1134168
Mouse monoclonal anti-IgM PE (clone AF6-78)	Biolegend	Cat#406208; RRID:AB_315041
Rat monoclonal anti-IgD Pacific Blue(clone 11-26c.2a)	Biolegend	Cat#405711; RRID:AB_1937245
Rat monoclonal anti-CD86 PE/Cy7 (clone GL-1)	Biolegend	Cat#105014; RRID:AB_439783
Rat monoclonal anti-CXCR4 PE (clone L276F12)	Biolegend	Cat#146505; RRID:AB_2562782
Rat monoclonal anti-CXCR5 biotin (clone 2G8)	BD Biosciences	Cat#551960; RRID: AB_394301
Rat monoclonal anti-Foxp3 PerCP/Cy5.5 (clone FJK-16 s)	Thermo	Cat#45-5773-80; RRID: AB_914349
Rat monoclonal anti-IL-4 PE (clone 11B11)	Biolegend	Cat#504104; RRID: AB_315318
Rat monoclonal anti-IL-21 PE (clone mhalx21)	Thermo	Cat#12-7213-82; RRID: AB_1834466
Armenian Hamster monoclonal anti-CD3 (clone 145-2C11)	Biolegend	Cat#100314; RRID: AB_312679
Syrian hamster monoclonal anti-CD28 (clone 37.51)	Biolegend	Cat#102112; RRID: AB_312877
Mouse monoclonal anti-TGFβ (clone 1D11.16.8)	BioXcell	Cat#BE0057; RRID: AB_1107757
Rat monoclonal anti-IFNγ (clone XMG1.2)	BioXcell	Cat#BE0055; RRID: AB_1107694
Rat monoclonal anti-IL4 (clone 11B11)	BioXcell	Cat#BE0045; RRID: AB_1107707
Rat monoclonal anti-IL12 p40 (clone C17.8)	BioXcell	Cat#BE0051; RRID: AB_1107698
Goat polyclonal anti-mouse IgG (H+L) Peroxidase	ZSGB-BIO	Cat# ZB-2305; RRID: AB_2747415
Rat monoclonal anti-Bcl-6 PE (clone 7D1)	Biolegend	Cat#358504; RRID: AB_2562152
Goat polyclonal anti-Rat IgG-Fc Alkaline Phosphatase	BETHYL	Cat# A110-136AP; RRID: AB_230270
Monoclonal anti-CD278-ICOS (clone C398-4A)	Thermo	Cat#16-9949-82; RRID: AB_469296
Rabbit polyclonal anti-Foxo3	Abcam	Cat#12162; RRID: AB_298893
Rat monoclonal anti-CD45R/B220 (clone RA3-6B2)	BD Biosciences	Cat# 550286; RRID: AB_393581

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mouse monoclonal anti-Bcl-6 (clone IG191E/A8)	Biolegend	Cat# 648301; RRID: AB_2274637
Chemicals, Peptides, and Recombinant Proteins		
Erythrocyte lysis buffer	QIAGEN	Cat#79217
Imject® Alum Adjuvant	Thermo	Cat# 77161
Ovalbumin	Sigma	Cat# A5503
7-AAD	BD Biosciences	Cat#559925
Neomycin trisulfate salt hydrate	Sigma	Cat#N6386
Difco™ Skim Milk	BD Biosciences	Cat#252100
Streptavidin-APC	Biolegend	Cat#405207
Annexin V-PE	Biolegend	Cat#640908
Recombinant Murine IL-6	Peprotech	Cat#216-16
Recombinant Murine IL-21	Peprotech	Cat#210-21
Critical Commercial Assays		
RNeasy mini-kit	QIAGEN	Cat#74104
PrimeScript® RT reagent Kit	TAKARA	Cat#DRR037A
Power SYBR Green master mix	Thermo	Cat#4367659
IL-21 Mouse Uncoated ELISA Kit	Invitrogen	Cat#88-8210-22
DouMaxVision™ Immunohistology Double-stain Kit	MXB biotechnologies	Cat#KIT-9998
BCIP/NBT Alkaline phosphatase Color Development Kit	Beyotime Biotechnology	Cat#C3206
Experimental Models: Organisms/Strains		
Mouse: *Rag1*: B6.129S7-*Rag1*^{tm1Mom}/J	The Jackson Laboratory	JAX: 002216
Mouse: *Foxo3*$^{-/-}$: B6.*EGE-YQL-010*^{tm1Rdp}	Biocytogen	N/A
Mouse: B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ	The Jackson Laboratory	JAX: 002014
Oligonucleotides		
See Table S1 for Primers used for real-time PCR, genotype and ChIP assay	N/A	N/A
Software and Algorithms		
FlowJo V10 software.	BD Biosciences	https://www.flowjo.com/
Prism 5.0 software	GraphPad	https://www.graphpad.com/scientific-software/prism/
Other		
Mouse T cell enrichment columns	R&D	Cat#MTCC-25

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Dong Zhang (zhangd@ccmu.edu.cn).

Materials Availability

Mouse strains generated in this study have been deposited to the the Jackson Laboratory (JAX002216 (*Rag1*$^{-/-}$) and JAX002014(CD45.1)) and the experimental animal center of Beijing Friendship Hospital (*Foxo3*$^{-/-}$). This study did not generate new unique reagents.

Data and Code Availability

This study did not generate any datasets or codes.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Foxo3 knockout (*Foxo3*$^{-/-}$) mice were generated by Biocytogen Inc. using a CRISPR/Cas9 based approach. Eight-week old male *Foxo3*$^{-/-}$ mice and their wild-type (WT) littermates were used in this study. C57BL/6 *Rag1*$^{-/-}$ mice were purchased from the Jackson Laboratory. Male mice aged 8-12 weeks were used in all experiments. Mice were maintained in a pathogen-free, temperature-controlled environment under a 12-hour light/dark cycle at Beijing Friendship Hospital, and all animal protocols were approved by the Institutional Animal Care and Ethics Committee of Beijing Friendship Hospital.

METHOD DETAILS

Cell isolation

Single cells were isolated from the spleen and lymph nodes. Red blood cells were lysed in lysis buffer (QIAGEN). Mouse T cell enrichment columns (R&D) were used for enrichment of splenic and lymph node T cells. Enriched T cells were further sorted for CD4$^+$CD25$^-$ T cells by FACS Aria II (BD Bioscience). The purity of sorted cells was > 97%.

Flow cytometry

Lymphocytes were stained with combinations of anti-CD4-Pacific Blue (GK1.5), anti-B220-FITC (RA3-6B2), anti-PD-1-PE or PE/Cy7 (RMP1-30), Streptavidin-APC, anti-CD69-PE/Cy7 (H1.2F3), anti-CD45-PE/Cy7 (30-F11), anti-CD19-FITC (6D5), anti-GL-7-PE or PerCP/Cy5.5 (GL7), anti-Fas-APC (SA367H8), anti-CD138-BV421 (281-2) anti-CD45.1-PE/Cy7 (A20), anti-IgM-PE (AF6-78), anti-IgD-Pacific Blue (11-26c.2a), anti-CD86-PE/Cy7 (GL-1), anti-CXCR4-PE (L276F12), anti-Bcl6-PE (7D1), and Annexin V-PE (Biolegend). Anti-CXCR5-biotin (2G8), anti-IgG1-APC (A85-1), and 7-AAD were purchased from BD Bioscience. Anti-Foxp3-PerCP/Cy5.5 (FJK-16 s) was purchased from eBioscience. Anti-IL-4 (11B11), anti-IL-21 (mhalx21) antibodies, and intracellular staining kits (Biolegend) were used to detect intracellular cytokines according to the manufacturer's instructions. Samples were detected on a FACS Aria II. Data were analyzed using FlowJo V10 (TreeStar) software.

Generation of Tfh *in vitro*

Generation of Tfh cells from CD4 T cells was described previously (Rumble and Segal, 2014). Briefly, CD4$^+$CD25$^-$ T cells were isolated from splenocytes of WT or *Foxo3*$^{-/-}$ mice. Purified CD4$^+$CD25$^-$ T cells were then cultured with anti-CD3 (3 μg/ml, anti-CD28 (2 μg/ml), IL-6 (100 ng/ml, Peprotech), IL-21 (50 ng/ml, Peprotech), anti-TGFβ (20 μg/ml, R&D System), anti-IFNγ (10 μg/ml, BioXcell), anti-IL4 (10 μg/ml, R&D System) and anti-IL12 (10 μg/ml, Peprotech) for 6 days. On day 7, converted CD4$^+$CXCR5$^+$PD-1$^+$ Tfh cells were detected and sorted through FACS Aria II (BD Biosciences).

Adoptive transfer experiments

For adoptive transfer experiments, naive CD4$^+$CD25$^-$ T cells and CD19$^+$ B cells were sorted by FACSAria II (BD Bioscience). *Rag1*$^{-/-}$ mice were injected with 4 × 10^6 CD4$^+$CD25$^-$ T cells (from WT or Foxo3$^{-/-}$ mice) plus 7 × 10^6 B cells (from WT mice) through the tail vein. Then, at day 1, day 14 and day 28, recipient mice were immunized intraperitoneally (i.p.) with 20 μg OVA (Sigma) and 100 μL Alum Adjuvant (Thermo). Seven days after the last immunization, *Rag1*$^{-/-}$ mice were euthanized for further investigation.

Bone marrow chimera

Lethally irradiated CD45.1 recipients were prepared by exposure to 900 rad (Gammacell® 1000 Elite). Irradiated mice were given 2mg/mL neomycin (Sigma) in their drinking water. Donor bone marrow (*Foxo3*$^{-/-}$, WT or 45.1) was isolated from the tibia and femur of 8-week-old mice and resuspended in 1640 medium. Bone marrow cells were flushed from bones by 22-G needle and passed by 40-μm strainer. Each irradiated recipient was given 2x10^7 bone marrow cell from *Foxo3*$^{-/-}$ or WT and CD45.1 mice at ratio of 1:1 intravenously. OVA protein vaccination was processed after 6 weeks as described above.

Quantitative real-time PCR

Total RNA was extracted from cells using the RNeasy mini-kit (QIAGEN) and reverse transcribed into cDNA using the PrimeScript® RT reagent Kit (TAKARA). Real-time quantitative polymerase chain reaction was performed with Power SYBR Green master mix (Applied Biosystems), and gene amplification was performed on the ABI 7500 Sequence Detection System (Applied Biosystems). Gene-specific primers used for specific genes and β-actin are shown in Table S1. Relative gene expression was quantitatively analyzed by the comparative Ct method ($2^{-\Delta\Delta CT}$). Data were normalized to β-actin mRNA levels.

Measurement of OVA-specific IgG and IL-21

Levels of OVA-specific IgGs were measured using ELISA. Ninety-six-well plates were coated with 0.5 μg/ml OVA protein (Sigma) at 4°C overnight. Then, plates were blocked with 1% milk (BD Bioscience)/PBS for 1 h at 37°C. After washing with PBST, 1: 2000 – 1:128000 diluted serum was used to incubate samples for 2 h at 37°C. Plates were then washed 6 times and incubated with HRP-labeled goat anti-mouse IgG (ZSGB-BIO) at a dilution of 1:1000 for 30 min at room temperature (RT). TMB substrates were

added after washing with PBST, and reactions were stopped with HCl solution. IL-21 levels in serum and supernatant were quantitated using a Mouse IL-21 ELISA Kit (Invitrogen) as per the supplier's protocol. Optical density was measured at 450 nm.

Immunohistochemistry

Spleen and lymph nodes were fixed in formalin prior to sectioning. Four micron thick sections were cut and stained. For GC identification, 4 μM LNs and spleen sections were stained with anti-Bcl6 (Biolegend, diluted 1:400) and anti-B220 (BD Bioscience, diluted 1:10) antibodies. Secondary antibodies, anti-mouse IgG-HRP (MXB biotechnologies) and Anti-Rat IgG-AP (BETHYL), were used to amplify the detection signal. A DAB Substrate Kit (MXB biotechnologies) and BCIP/NBT Alkaline phosphatase Color Development Kit (Beyotime Biotechnology) were used according to the manufacturer's protocol.

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) assay was performed according to the SimpleChIP® Plus Enzymatic Chromatin IP Kit's instructions (New England Biolabs). $CD4^+$ T cells were fixed, sonicated, and collected for ChIP assay. DNA fragments were immunoprecipitated with antibodies specific to Foxo3a (Abcam) or control rabbit IgG at 4°C overnight. Subsequently, DNA fragments were decrosslinked, purified from the complexes, and ethanol-precipitated. Immunoprecipitated chromatin was amplified by primers (Table S1) corresponding to specific regions of the Foxo3 genomic locus. Meanwhile, immunoprecipitated DNA fragments were detected by quantitative real-time PCR. To calculate fold enrichment of precipitated IL-21, each sample was normalized to the corresponding input. All ChIP assays were performed in triplicate.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis was performed using Prism 5.0 software (Graph Pad Software). Values are expressed as the mean ± SEM. Analyses of significant differences were performed using Student's t test and one-way analysis of variance. P-values < 0.05 were considered significant.