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ICOS Coreceptor Signaling Inactivates the Transcription Factor FOXO1 to Promote Tfh Cell Differentiation

Highlights
- ICOS signaling transiently inactivates FOXO1 to generate Tfh cells
- FOXO1 regulates a Tfh cell gene program exemplified by negative regulation of Bcl6
- Enforced nuclear localization of FOXO1 prevents Tfh cell differentiation
- FOXO1 promotes final GC-Tfh cell differentiation

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In Brief
How ICOS signaling causes the induction of BCL6 leading to Tfh cell differentiation is incompletely understood. Hedrick and colleagues show that ICOS signaling transiently inactivates FOXO1, which in turn relieves FOXO1-dependent inhibition of BCL6 expression and Tfh differentiation. In contrast, FOXO1 promotes late-stage germinal center-Tfh cell differentiation.
ICOS Coreceptor Signaling Inactivates the Transcription Factor FOXO1 to Promote Tfh Cell Differentiation

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SUMMARY

T follicular helper (Tfh) cells are essential in the induction of high-affinity, class-switched antibodies. The differentiation of Tfh cells is a multi-step process that depends upon the co-receptor ICOS and the activation of phosphoinositide-3 kinase leading to the expression of key Tfh cell genes. We report that ICOS signaling inactivates the transcription factor FOXO1, and a Foxo1 genetic deletion allowed for generation of Tfh cells with reduced dependence on ICOS ligand. Conversely, enforced nuclear localization of FOXO1 inhibited Tfh cell development even though ICOS was overexpressed. FOXO1 regulated Tfh cell differentiation through a broad program of gene expression exemplified by its negative regulation of Bcl6. Final differentiation to germinal center Tfh cells (GC-Tfh) was instead FOXO1 dependent as the Foxo1−/−GC-Tfh cell population was substantially reduced. We propose that ICOS signaling transiently inactivates FOXO1 to initiate a Tfh cell contingency that is completed in a FOXO1-dependent manner.

INTRODUCTION

The generation of high-affinity antibodies requires naive CD4+ T cells to sequentially be activated, proliferate and differentiate, acquire proximity to the B cell follicles, and provide B cells with “help” in the form of antigen-specific interactions, co-receptor binding, and cytokine signaling. These specialized CD4 cells have been termed T follicular helper (Tfh) cells, and they are essential to promote the germinal center (GC) reaction including B cell expansion, class switching, selection, and development of high-affinity antibody-forming cells (Liu et al., 2013; Crotty, 2014; Ueno et al., 2015). In the past several years, much has been learned about Tfh cell differentiation; however, the cellular programming leading to this state remains incompletely understood.

Inducible T cell co-stimulator (ICOS) is a potent co-receptor distinct from CD28 that is induced on activated T cells and highly expressed on Tfh cells. ICOS signaling is necessary for complete GC development, T cell-dependent B cell help, and antibody class switching (Vinuesa et al., 2005), and this is due to a role for ICOS in the differentiation of activated T cells to Tfh cells (Ueno et al., 2015).

Tfh cell differentiation is a multi-step process that begins with dendritic cell priming and further requires B cells for additional differentiation and maintenance (Crotty, 2014; Ueno et al., 2015). The initial dendritic cell priming is sufficient to induce a CXCR5+BCL6+ Tfh cell, and this was found to be dependent on ICOS signaling (Qi et al., 2014). However, further ICOSL stimulation from B cells is required for the final differentiation and maintenance of GC-Tfh cells (Pepper et al., 2011; Crotty, 2014), and this is consistent with studies showing that ICOS is able to influence homing to GCs through the induction of filopodia (Franko and Levine, 2009; Xu et al., 2013). Signal transduction through ICOS results in the potent activation of phosphoinositide-3 kinase (PI3K), and this is a key event in Tfh differentiation (Rolf et al., 2010b). In a manner not yet understood, this leads to increased expression of BCL6, which has been described as an essential transcription factor for the differentiation and function of Tfh cells (Choi et al., 2013).

A major pathway downstream of PI3K signaling is the AKT-mediated inactivation of FOXO family transcription factors. AKT mediates the triple phosphorylation of FOXO proteins causing their nuclear egress (Calnan and Brunet, 2008). FOXO transcription factors are important for the expression of cyclin-dependent kinase inhibitors and proapoptotic molecules, and thus their inhibition is an essential aspect of growth factor-mediated cell-cycle progression and survival. In T cells, FOXO transcription factors have been shown to regulate multiple, specialized functions including the expression of the Il7ra and Klf2—control points for T cell survival and homing (Ouyang and Li, 2011; Hedrick et al., 2012). In addition, mice with a T cell-specific deletion of Foxo1 lack functional FOXP3+ Treg cells and spontaneously develop systemic autoimmunity. We previously
Figure 1. Loss of FOXO1 Amplifies Tfh Differentiation

(A and B) WT or Foxo1TKO OTII cells were transferred into CD45.1 hosts, immunized with OVA plus adjuvant, and spleen cells were analyzed days 4 post immunization. (A) The percentages of CXCR5lo, CXCR5int, and CXCR5hiBCL6+ cells (left) or PD1 expression (right) were determined by flow cytometry and (B) The numbers of each cell type in the spleen were calculated, representative of two independent experiments.

(C) Adoptive transfer similar to (A) above were carried out with Foxo1KO OTII cells and the percentages of WT or Foxo1KO CXCR5lo, CXCR5int, and CXCR5hiBCL6+ cells were determined days 4 post immunization.

(D) Expression of CXCR5, BCL6, and PD1 from WT (filled histogram) or Foxo1KO (open histogram) CXCR5int OTII cells days 4 post immunization (n = 3). Representative of two independent experiments.

(legend continued on next page)
noted that these mice accumulate a large population of Tfh cells, form GCs, and produce circulating, anti-DNA antibodies, and we proposed that the PI3K-AKT-FOXO1 signaling pathway controls lineage commitment that, in part, specifies the Treg versus Tfh alternative cell fates (Kerdiles et al., 2010; Hedrick et al., 2012). Though provocative, these experiments highlight a necessity to study the role of FOXO transcription factors in T cell differentiation without the complications of autoimmunity caused by an insufficiency of Treg cells. In support of this idea, a report recently appeared showing that the ubiquitin ligase, ITCH, facilitates Tfh differentiation, and indeed it appears to act through the degradation of FOXO1 (Xiao et al., 2014). Here, we test the proposition that ICOS signaling acts to initiate a program of Tfh differentiation through inhibition of FOXO1 and the resulting effects on gene expression. Specifically, the deletion of Foxo1 results in enhanced BCL6 expression and exaggerated differentiation of Tfh cells.

RESULTS

Loss of FOXO1 Amplifies Tfh Differentiation

In accord with the high prevalence of Tfh cells in mice with a T cell-specific Foxo1 deletion (Kerdiles et al., 2010), we tested whether ICOS-mediated Foxo1 inactivation constitutes an important step in Tfh cell differentiation. As such, we adoptively transferred Foxo1<sup>f/f</sup>Cd4Cre<sup>+</sup>CD45.2<sup>+</sup> (Foxo1<sup>TKO</sup>) OTII or Foxo1<sup>f/f</sup>CD45.2<sup>+</sup> (wild type, WT) OTII cells into CD45.1 mice. In this and subsequent experiments, the starting population was depleted of CD25<sup>+</sup>CD69<sup>+</sup> cells prior to transfer. Host mice were then immunized with OVA plus adjuvant. Four days post-immunization WT and Foxo1<sup>TKO</sup> OTII cells were fully activated as determined by CD44 expression (data not shown), and the WT OTII cells differentiated into three cell populations: CXCR5<sup>hi</sup>BCL6<sup>hi</sup> cells, described as T effector (Teff) cells; CXCR5<sup>int</sup> cells, Tfh cells; and CXCR5<sup>lo</sup>BCL6<sup>lo</sup> T cells that are destined to be GC-Tfh cells (Pepper et al., 2011; Liu et al., 2013). By contrast, almost all Foxo1<sup>TKO</sup> OTII cells displayed CXCR5<sup>int</sup>BCL6<sup>lo</sup> expression characteristic of Tfh cells (Figure 1A). Consistent with this, PD1 expression was also elevated in Foxo1<sup>TKO</sup> compared to WT T cells (Figure 1A).

Contrary to expectations given the role of FOXO transcription factors in the expression of Bim and Fas-ligand (Calnan and Brunet, 2008; Fu and Tindall, 2008), there was a decrease in the total number of Foxo1<sup>TKO</sup> T cells compared with WT (Figure S1A). The analysis of cultured T cells showed that this defect in accumulation was not due to retarded cell division, but rather, increased apoptosis (Figure S1B–1F). It is cell-intrinsic (Figure S1D), and could be completely rescued by the addition of a pan-caspase inhibitor (Figure S1F). Although activation via interleukin-2 (IL-2) or a superantigen leads to FOXO1 inactivation (Stahl et al., 2002; Fabre et al., 2005), an important point is that this inactivation was transient, such that at least by 24 hr post-activation, FOXO1 contributed to CD4<sup>+</sup> T cell survival.

All three populations were reduced with a Foxo1 deletion, although the decrease was minimal for Tfh (CXCR5<sup>int</sup>) cells (Figure 1B). IL-7 is required for naive T cell survival and normal expression of BCL2 in naive T cells, and it increases Tfh cell differentiation (Surh and Sprent, 2008; Seo et al., 2014). As Foxo1-deficient naive cells have reduced expression of IL-7Rα (Kerdiles et al., 2009), we determined whether enforced expression of Il7ra (Yu et al., 2004) would rescue survival or alter the course of the response. Results showed no effect of Il7ra expression on the proportion or number of Foxo1<sup>TKO</sup> cells that became Tfh cells (Kerdiles et al., 2010; data not shown).

A Foxo1 loss of function was further tested by acute deletion just prior to immunization. After treatment with tamoxifen, T cells were harvested from Foxo1<sup>1<sup>st</sup> Rosa26<sup>Cre-ERT2</sup> OTII (Foxo1<sup>TKO</sup>) and Rosa26<sup>Cre-ERT2</sup> OTII mice (WT) (Kerdiles et al., 2009) and transferred into naive hosts. The starting and unimmunized OTII populations from these mice were equivalent for the expression of CD44 and CXCR5 (Figure S1G and data not shown). Notably, the proportion of Foxo1<sup>TKO</sup> OTII cells that acquired a CD44<sup>hi</sup> activated phenotype day 4 post immunization was equivalent to WT, and yet similar to Foxo1<sup>TKO</sup> T cells, nearly all Foxo1<sup>TKO</sup> OTII cells displayed a CXCR5<sup>int</sup>BCL6<sup>lo</sup> phenotype (Figures 1C and S1H). Similar to Foxo1<sup>TKO</sup> T cells, further analysis of this CXCR5<sup>int</sup> Tfh subset revealed higher expression of CXCR5, BCL6, and PD1 in Foxo1<sup>TKO</sup> cells compared with the equivalent WT CXCR5<sup>int</sup> population (Figures 1D and S1H).

To determine whether these effects applied to other immunization conditions, we analyzed the response to infection with <i>Listeria monocytogenes</i>. After adoptive transfer of OTII cells, host mice were infected with actA-deficient <i>Listeria monocytogenes</i> (ΔActA-Lm) expressing OVA (Erret et al., 2009), and the analysis day 4 post infection revealed that virtually all the Foxo1<sup>TKO</sup> OTII cells were CXCR5<sup>+</sup> (Figure 1E). Again, within the CXCR5<sup>+</sup> population, Foxo1<sup>TKO</sup> T cells were uniformly higher by approximately two-fold for the expression of CXCR5, BCL6, and PD1 (Figure 1E).

A defining characteristic of Tfh cells is location within the B cell follicles, whereas the eponymous GC-Tfh cells are located within GCs. To analyze the role of FOXO1 in localization, we determined the expression of homing molecules in addition to CXCR5. As expected, based on the control of Klf2 by Foxo1 (Fabre et al., 2008; Kerdiles et al., 2009), virtually all Foxo1<sup>TKO</sup> OTII cells were CD62L<sup>–</sup> 4 days post immunization, whereas the WT T cells displayed heterogeneous expression (Figure 1F). CCR7 expression was unchanged with respect to activated WT T cells, but a proportion of the Foxo1<sup>TKO</sup> OTII cells were low for PSLGL1 (Figure 1F), a phenotype that allows T cells to exit the T cell zone (Crotty, 2014). Combined with the expression of CXCR5 (e.g., Figure 1D), Foxo1<sup>TKO</sup> OTII cells appear to express a repertoire of homing molecules that would promote homing to B cell areas of the spleen (Crotty, 2014).

WT or Foxo1<sup>TKO</sup> OTII T cells were directly examined 4 days after immunization by immunohistology. WT OTII cells were mostly found within the splenic T cell zone including some cells along the GCs, and produce circulating, anti-DNA antibodies, and we proposed that the PI3K-AKT-FOXO1 signaling pathway controls lineage commitment that, in part, specifies the Treg versus Tfh alternative cell fates (Kerdiles et al., 2010; Hedrick et al., 2012). Though provocative, these experiments highlight a necessity to study the role of FOXO transcription factors in T cell differentiation without the complications of autoimmunity caused by an insufficiency of Treg cells. In support of this idea, a report recently appeared showing that the ubiquitin ligase, ITCH, facilitates Tfh differentiation, and indeed it appears to act through the degradation of FOXO1 (Xiao et al., 2014). Here, we test the proposition that ICOS signaling acts to initiate a program of Tfh differentiation through inhibition of FOXO1 and the resulting effects on gene expression. Specifically, the deletion of Foxo1 results in enhanced BCL6 expression and exaggerated differentiation of Tfh cells.

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the T cell-B cell border. In contrast, a larger proportion of the Foxo1<sup>KO</sup> OTII cells was found in the follicle with relatively few cells found deep within the T cell zone (Figure S1I). However, we note that the Foxo1<sup>KO</sup> cells were also not found deep in the B cell follicle.

The Regulation FOXO1 and ICOS Is Coupled via a Negative Feedback Loop

To analyze the relationship between ICOS signaling and FOXO1, we tested whether ICOS signaling would inactivate FOXO1 via nuclear egress (Calnan and Brunet, 2008). Naive CD4<sup>+</sup>T cells expressing a FOXO1-GFP fusion protein were activated for 48 hr under iTfh conditions, rested for 24 hr, and restimulated for 30 min with antibody specific for CD3 in the presence or absence of agonist ICOS-specific antibody. At 30 min post-restimulation there was no difference in the amount of FOXO1-GFP in live cells (Figure 2A, left). However, upon restimulation through CD3 and ICOS, but not CD3 alone, the similarity score (ImageStream analysis) for DRAQ5 (nucleus) and FOXO1-GFP was reduced; this corresponds with reduced co-localization and nuclear FOXO1 (Figure 2A, middle). In agreement, there was an increased percentage of cells stimulated through ICOS that displayed FOXO1-GFP exclusively in the cytoplasm (Figure 2A, right). However, at 24 hr post restimulation through CD3 and ICOS, the amount of FOXO1-GFP was increased with little difference in the DRAQ5, FOXO1-GFP similarity score (Figure 2B, left, middle). Consistent with these results, nuclear intensity of FOXO1-GFP was not diminished in live cells 24 hr post-restimulation through CD3 and ICOS (Figure 2B, right). These observations show nuclear FOXO1, which was lost at 30 min post-activation, was reestablished by 24 hr.

FOXO transcription factors have been shown to positively regulate the transcription of growth factor receptors (e.g., IL-7Rα, insulin receptor) that, in turn, signal through PI3K to cause FOXO inactivation (Hedrick, 2009; Kerdiles et al., 2009). This creates a negative feedback loop. Activation through CD3 and CD28 induced ICOS expression in WT T cells, and this induction was attenuated in Foxo1<sup>KO</sup> T cells (Figure 2C, left). Because ICOS signaling also inactivated FOXO1, how is ICOS maintained in differentiating Tfh cells? To examine this, we further measured ICOS signaling also inactivated FOXO1, how is ICOS maintained under iTfh conditions? To examine this, we analyzed the dependence of Tfh differentiation on ICOSL in two ways. In one set of experiments, we transferred WT or Foxo1<sup>KO</sup> T cells in the presence or absence of antibodies specific for ICOSL. In a second set of experiments, we transferred T cells into WT or ICOSL<sup>−/−</sup> hosts. In these experiments, the results were similar. Although the presence of WT Tfh cells displayed a strong dependence on ICOSL recognition, this dependence was greatly reduced for Foxo1<sup>KO</sup> T cells (Figure 3A–3D). Importantly, in both experimental models, the number of Foxo1<sup>KO</sup> CXCR5<sup>+</sup> OTII cells was substantially greater than the number of WT CXCR5<sup>+</sup> cells under these conditions (Figures 3B and 3D). In particular, although the differentiation of WT cells was virtually lost in Icos<sup>−/−</sup> hosts (Figures 3C and 3D) (Choi et al., 2011; Pepper et al., 2011), in the absence of FOXO1 the mean number of CXCR5<sup>+</sup> T cells was increased by 10-fold over WT controls (Figure 3D). Further experiments showed that CXCR4 induction, shown to have a stringent requirement for ICOS in WT T cells (Odegard et al., 2008) was induced in Foxo1<sup>KO</sup> T cells in an ICOSL-independent manner (Figures S3A and S3B). From these data, we conclude that loss of FOXO1 facilitates differentiation into Tfh cells with a greatly diminished requirement for ICOS signaling, i.e. FOXO1 inactivation is epistatic to ICOS expression and signaling.

Loss of FOXO1 Promotes B Cell Help and Anti-DNA Antibodies in the Absence of ICOS

To determine whether loss of Foxo1<sup>KO</sup> could complement a loss of Icos, we bred Foxo1<sup>T<sub>KO</sub></sup> mice and analyzed the proportion of CXCR5<sup>+</sup>PD1<sup>+</sup> cells from each of four genotypes. To account for the increase in activated CD4 cells in the Foxo1<sup>T<sub>KO</sub></sup> mice and the reduced population of activated cells in Icos<sup>−/−</sup> mice (Odegard et al., 2008; Kerdiles et al., 2010), we focused on the activated CD4<sup>+</sup> (CD44<sup>+</sup>) population. In addition, we enumerated class-switched and GC B cells. For each of these parameters, the deficiencies displayed by Icos<sup>−/−</sup> mice were all or partially rescued by the inclusion of the Foxo1<sup>T<sub>KO</sub></sup> alleles.
Although little to no immunoglobulin G (IgG) isotype anti-DNA antibodies were detected in the Icos−/− mice, significant titers were measured in DKO mice (Figure 4D, left). DKO mice also had significantly higher levels of total IgG levels in the sera than Icos−/− mice (Figure 4D, right). The presence of GCs and isotype switched antibodies was not simply due to a lack of regulatory FOXP3+ Tfh (Tfr) cells, because the frequency of the CXCR5+ Tfr population within the Treg population was not reduced with the deletion of Foxo1 (Figure 4E). These data indicate that deletion of Foxo1 in T cells is sufficient to allow differentiation of a Tfh-like cell in the absence of ICOS, and these cells cooperate with B cells to produce isotype-switched, anti-DNA antibodies—at least in the absence of effective Treg cells.

**FOXO1 Negatively Regulates BCL6 Expression**

If the loss of FOXO1 is important for Tfh differentiation, then a prediction is that FOXO1 inhibition as a consequence of ICOS signaling will facilitate the induction of BCL6 expression (Choi

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**Figure 2. The Regulation FOXO1 and ICOS Is Coupled via a Negative Feedback Loop**

(A) Plots show MFI of FOXO1-GFP (left), similarity score between DRAQ5 and FOXO1-GFP (Middle), and the percent of cells with FOXO1-GFP exclusively in the cytoplasm (right) 30 min post restimulation through CD3 and ICOS. None indicates the cells were not restimulated. Data are representative of two independent experiments. (B) Plots show MFI of total FOXO1-GFP (left), similarity score between DRAQ5 and FOXO1-GFP (middle), and intensity of FOXO1-GFP overlapping with the nuclear mask (right) 24 hr post restimulation. (C) ICOS expression on WT or Foxo1KO CD4 cells activated in vitro for 72 hr via CD3 and CD28 in the presence or absence of Tfh conditions. The histograms show the expression of ICOS on cells activated with anti-CD3 and anti-CD28 without the addition of exogenous cytokines (left). Data are representative of two independent experiments. (D) Histograms depict ICOS expression on CXCR5lo, CXCR5int, or CXCR5hiBCL6+ from WT or Foxo1KO OTII cells days 4 post immunization. Data are representative of two independent experiments. (E) Analysis of FOXO1-specific ChIP-Seq of naive CD4 T cells. The most frequent consensus binding site was determined to be TGTTCAC, the size of the nucleotide in the graphic corresponds with its frequency. (F) The Icos locus is shown for FOXO1-specific ChIP-seq (top track) (see also Figure S2B), and the centrally positioned nucleotide sequence within the promoter peak is listed. The bottom track shows mammalian sequence conservation (UCSC Genome Browser). (G) FOXO1-specific ChIP of Icos locus from WT CD4 T cells activated in vitro.
et al., 2011). In naive cells, the low amount of BCL6 detected was unchanged between WT and Foxo1KO mice (data not shown). T cells were activated for 48 hr under iTfh conditions, and they were rested for 24 hr and re-stimulated with or without ICOS-specific antibody for a further 24 hr. Restimulation through ICOS increased BCL6 expression, whereas it was substantially higher in Foxo1KO T cells compared with WT T cells under all conditions (Figure 5A). In particular, Foxo1KO T cells re-stimulated through CD3 alone expressed more BCL6 than WT T cells stimulated through CD3 and ICOS. There was a further induction of BCL6 in the Foxo1KO T cells stimulated through ICOS (compare anti-CD3 with anti-CD3 plus anti-ICOS), and this suggests that an additional pathway downstream of ICOS might play a role in BCL6 induction. Similar to protein expression, WT Bcl6 RNA increased upon restimulation in the presence of anti-ICOS, and it was expressed in higher amounts in Foxo1KO cells compared to WT cells (Figure 5B). Furthermore, this increase in BCL6 was cell-intrinsic (Figure 5C), and it was not secondary to selective death of Foxo1KO T cells (Figure S4A).

Analysis of FOXO1 binding by ChIP-Seq in naive CD4 T cells showed that FOXO1 is exclusively bound to the Bcl6 locus at the boundary of the first (38 bp) non-coding exon and the first intron (Figures 5D and S4B). This region includes tandem sequences separated by 30 bases that are very similar to the conserved FOXO1 consensus site (Figures 5D and S4B), and we have also found this peak in naive and activated CD8 T cell data sets (data not shown). This region is highly conserved between mice and human beings and this conservation extends to a comparison of marsupials and eutherian mammals, implying evolutionary selection for at least 130 million years (Figure S4C).

FOXO1 binding to this site in naive T cells was confirmed by ChIP analysis (Figure 5E). We further examined whether FOXO1 binding is lost under conditions of T cell stimulation. After 48 hr of iTfh activation, cells were rested for 24 hr and tested (None), or re-stimulated through CD3 and ICOS for 1 hr or 24 hr. As shown, FOXO1 was bound to this site in T cells activated under iTfh conditions, but it was reduced upon restimulation with through CD3 and ICOS (Figure 5F). This is consistent with the initially reduced nuclear localization of FOXO1 (Figure 2A). However, nuclear FOXO1 is not decreased 24 hr post-restimulation through CD3 and ICOS (Figure 2B), and yet binding of FOXO1 to Bcl6 was still reduced (Figure 5F). These data are consistent with FOXO1 binding to the Bcl6 gene and mediating transcriptional repression that is relieved upon ICOS signaling; however, we lack direct evidence for transcription repression...
that might include germline mutations in the tandem FOXO1 binding sites.

Further analysis of the 4333 FOXO1 genomic binding sites revealed many genes involved in Tfh differentiation located within close proximity. Inspection of Cxcr5, Batf, Cor7, Cxcr4, Irf4, Selplg (P-selectin ligand-CD162), and Maf loci revealed one or more strong FOXO1 binding sites located near the transcriptional start site or within several kilobases (Figure S5). The exception was Maf, which is functionally important for terminally differentiated GC-Tfh cells (Liu et al., 2013).

Enforced Nuclear Localization of FOXO1 Prevents Tfh Differentiation

If FOXO1 inactivation is required for Tfh differentiation, enforced nuclear localized would be predicted to block the appearance of Tfh cells. To test this, we transduced T cells from OTII Foxo1AAA mice with a Hit and Run CRE recombinase retrovirus and adoptively transferred them (Silver and Livingston, 2001; Ouyang et al., 2012). After immunization, Foxo1AAA T cells expressed CD44+ (data not shown) and displayed superinduction of ICOS (Figure 6A) consistent with the importance of FOXO1 in the regulation of the Icos gene. Despite this, Foxo1AAA T cells displayed a reduced ability to differentiate into the Tfh phenotype as compared to WT (Figure 6B). The accumulation of Foxo1AAA T cells was also reduced (data not shown), and the origin of this defect is a topic of further investigation.

Foxo1KO T Cells Have Reduced Ability to Differentiate into GC-Tfh Cells

To characterize the role of FOXO1 in GC-Tfh differentiation, we examined a polyclonal response to L. monocytogenes. For this, we generated mixed WT:Foxo1TKO bone-marrow chimeras (Kerdiles et al., 2010). Mice were infected with ΔActA-Lm, and at day 9 the CXCR5int (and total CXCR5+ cells) cells were overrepresented within the Foxo1TKO population compared with WT cells. Surprisingly there was a notable paucity of Foxo1TKO CXCR5hiBCL6hi GC-Tfh cells (Figure 7A).

Studies have shown that MAF is an important transcription factor in Tfh development, and in particular, it might be essential for IL-4 expression associated with GC-Tfh cells (Liu et al., 2013; Crotty, 2014; Ueno et al., 2015). Consistent with this, analysis of WT OTII T cells 4 days after activation in vivo revealed that only the CXCR5hi PD1hi subset expressed high amounts of MAF (Figure 7B), and this was abrogated by treatment of the mice with anti-ICOSL (Figure 7C). Compatible with the lack of a BCL6hi population at GC time points, Foxo1TKO T cells were selectively deficient in the MAF+ population at both day 4 and day 7 post immunization (Figure 7D). In addition, Foxo1TKO OT1 T cells did not give rise to CXCR5hiBCL6hi or CXCR5hiPD1hi cells day 7 post immunization (Figure 7E), and at this time, FOXO1 is consistently expressed (Figure 7F). Similar results were found following infection with VSV-OVA (Figures 7G and 7H). In sum, these data show...
that genetic inactivation of Foxo1 exaggerates the differentiation of Tfh cells in an ICOS-independent manner, and yet, FOXO1 plays a role in the final differentiation to GC-Tfh cells.

**DISCUSSION**

Previous work established an early role for ICOS and its activation of PI3K signaling in the differentiation of CD4 T cells into Tfh cells, and this signaling pathway influences the induction of key molecules including BCL6, MAF, IL-4, and IL-21 (Bauquet et al., 2009; Gigoux et al., 2009; Rolf et al., 2010a; Choi et al., 2011). Since the basis for PI3K regulation of cell growth and differentiation largely emanates through AKT-mediated inhibition of FOXO1 transcriptional activity (Calnan and Brunet, 2008), we wished to test the idea that Foxo1 is epistatic to Icos in the elaboration of one or more of these Tfh characteristics. Additionally, two recent papers suggest that reduced expression of FOXO1, either due to increased expression of ICOS induced by loss of FOXP1, or due to ITCH-mediated degradation, may increase Tfh differentiation (Wang et al., 2014; Xiao et al., 2014). The studies described in this report provide a mechanism for those findings.

Tfh cells at the B-follicular border express CXCR5 and BCL6 (Ramiscal and Vinuesa, 2013), whereas GC-Tfh cells can be characterized by MAF expression. Here we show that deletion of Foxo1 exaggerated the initial antigen-driven step in Tfh differentiation resulting in an expanded proportion of CXCR5+ CD4 T cells localized to the border of B cell follicles. Foxo1 KO T cells were proportionately overrepresented as CXCR5 int BCL6 int cells, and in addition these Tfh cells expressed amounts of CXCR5, BCL6, PD1, and CXCR4 greater than those of the equivalent WT Tfh populations—although not to the level characteristic of GC-Tfh cells. In fact, in the absence of FOXO1, despite the increased proportion of Tfh cells, few GC-Tfh cells emerged.
even as late as 9 day post ActA-Lm infection. Our conclusion is that a transient inactivation of FOXO1 skews the contingency of effector versus Tfh differentiation, whereas progression to mature GC-Tfh cells is promoted by FOXO1.

We emphasize that FOXO1 inactivation is only transient. In T cells stimulated through CD3 and ICOS, nuclear FOXO1-GFP is reduced at 30 min but reestablished within 24 hr. Moreover, FOXO1 is required for T cell viability as early as 24 hr post activation. Whether there are mechanisms opposing AKT signaling or desensitizing ICOS signaling is not known; however, stress kinase phosphorylations, glycosylation, or methylation have all been shown to encourage nuclear location of FOXO factors (Hedrick et al., 2012). This is further illustrated by regulation of ICOS. Although FOXO1 clearly has a role for full ICOS expression, ICOS is induced early in DC-mediated antigen presentation, and remains high in Tfh and GC-Tfh cells despite its potential for signaling via PI3Kδ and causing negative feedback inactivation of FOXO1. Thus, although genetic ablations presented here and elsewhere point to an important contingency-based inactivation of FOXO1 (Wang et al., 2014; Xiao et al., 2014), they do not recapitulate the dynamics of FOXO1 inactivation. Furthermore, FOXO1 appears to be required for GC-Tfh differentiation, although the mechanism of action is unknown. The reduced expression of ICOS might limit the ability of Foxo1 KO Tfh cells to generate filopodia, which allow for Tfh cells to home from the T-B border to the GC (Xu et al., 2013). This possibility would be consistent with the presence of GC-Tfh cells in Foxo1 TKO mice contrasted with the loss of Foxo1 TKO GC-Tfh cells in competition with WT cells. FOXO1 has also been shown to bind to the Ifng locus and inhibit expression of IFN-γ (Ouyang et al., 2012), and thus in its absence, ectopic gene expression might subvert GC-Tfh differentiation. The most parsimonious explanation is that FOXO1 directly regulates the transcription of genes required for full GC-Tfh differentiation.

A complication described here is the observation that activated Foxo1 KO−/− CD4 T cells have a reduced viability compared to WT T cells. This raised the possibility that the increase in the proportion of Tfh cells could be due to selective death of Teff cells; however, the results show that this alone cannot explain the phenotype of Foxo1 KO−/− T cells. If the exaggerated proportion of Tfh cells were due only to preferential loss of Teff cells, then there would be no reduction in the requirement for ICOS signaling. In two different types of experiments we show that Foxo1 KO T cells differentiate into Tfh cells with a substantially reduced requirement for ICOS signaling. Similarly, the induced expression of BCL6 is an important part of the Tfh program, and loss of FOXO1 results in the increased expression of BCL6 compared to wild-type, even when apoptosis is blocked. In a separate line of experimentation, loss of Foxo1 genetically complemented the loss of Icos in that there emerged CXCR5+PD1+ cells, GC-B cells and anti-DNA IgG antibodies. In addition, a role for FOXO1 in Tfh differentiation is supported by the known signaling pathway downstream of ICOS in T cells, that is, PI3K and AKT activation (Rolf et al., 2010b), which was shown here to result in the inactivation of FOXO1. Finally, enforced nuclear expression of FOXO1 inhibits the differentiation Tfh cells, and the sum of these results provide a mechanism by which ITCH-mediated FOXO1 degradation is required for Tfh differentiation (Xiao et al., 2014).

These results demonstrate that inactivation of FOXO1 is an essential outcome of ICOS signaling in the contingency of CD4 T cell differentiation, and this establishes an important link in the signaling from ICOS to the induction of Bcl6 expression. Previous studies have reported that FOXO1, FOXO3, or FOXO4
Figure 7. FOXO1 KO T Cells Have Reduced Ability to Differentiate into GC-Tfh Cells

(A) Mixed bone-marrow chimeras (WT-CD4.1 and Foxo1TKO) were infected with ActA-Lm. CD4+B220-CD44hiLLO+ cells were phenotyped from each donor. Representative of two separate experiments. (B) MAF expression within CXCR5lo, CXCR5int, and CXCR5hiPD1hi OTII T cells donor. Representative of two separate experiments. (C) Percentages of CXCR5lo, CXCR5int, and CXCR5hiMAFhi subsets within WT Tfh cells day 4 post immunization (n = 3). Representative of three independent experiments. (D) Analysis similar to (C) using WT or Foxo1TKO OTII cells assayed at day 4 (n = 3–5) and day 7 (n = 3). One of at least two representative experiments.

Foxo1KO OTII cells assayed at day 4 and day 7 post immunization. One of at least two representative experiments.

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For in vivo Tfh cell experiments in which mice were immunized, OTII cells were adoptively transferred into CD45.1 hosts unless otherwise indicated. CD45.1 mice were used as hosts for adoptive transfer experiments, 10,000 cells of each WT OTII (CD45.1.2) and Foxo1TKO OTII (CD45.2) cells were transferred into the same host mice and the next day mice were infected with 10^6 pfu of VSV-OVA. Phenotype of transferred cells was determined 6–7 days post infection by flow cytometry.

In Vitro ICOS Signaling Experiments

To study ICOS signaling, we activated and restimulated cells with anti-ICOS similarly to previously described ICOS restimulation conditions (Rolf et al., 2010a). Briefly, WT or FoxO1-GFP naive CD4 (CD69 CD25 CD4+) T cells were purified by negative depletion and activated with anti-CD3 (2C11), 1 μg/ml anti-CD28 plus or minus 10 μg/ml anti-IFN-γ, 10 μg/ml anti-IL-4, 50 ng/ml IL-6, and 10 ng/ml IL-21 (Tfh conditions) in RPMI 10 for 48 hr. After 48 hr, the cells were rested in RPMI 10 for 24 hr. Following the rest, the cells were restimulated with soluble anti-CD3 0.5 μg/ml, goat anti-hamster 20 μg/ml (Vector Labs, Burlingame, CA) with or without stimulatory 2 μg/ml anti-ICOS (Clone: C398.4A, eBioscience). To determine whether ICOS signaling inactivated, we collected FOXO1 cells at 30 or 24 hr post restimulation and analyzed FOXO1-GFP compared to DRAQ5 staining using AMNIS ImageStream and BD LSR Fortessa analysis. To determine whether ICOS signaling through FOXO1 might be involved in ICOS upregulation of BCL6, we left WT or Foxo1TKO CD4 T cells in culture for 24 hr post restimulation and analyzed expression of Tfh markers by flow cytometry.

Imaging Flow Cytometry

FOXO1-GFP localization was determined using the 6X objective on ImageStreamX MkII (Amnis/EMD Millipore). FOXO1-GFP signal was compared to the nuclear mask generated using signal from DRAQ5 (Cell Signaling). Data was analyzed with IDEAS software including the nuclear localizer. To study ICOS signaling, we activated and restimulated cells with anti-ICOS similarly to previously described ICOS restimulation conditions (Rolf et al., 2010a). Briefly, WT or FoxO1-GFP naive CD4 (CD69 CD25 CD4+) T cells were purified by negative depletion and activated with anti-CD3 (2C11), 1 μg/ml anti-CD28 plus or minus 10 μg/ml anti-IFN-γ, 10 μg/ml anti-IL-4, 50 ng/ml IL-6, and 10 ng/ml IL-21 (Tfh conditions) in RPMI 10 for 48 hr. After 48 hr, the cells were rested in RPMI 10 for 24 hr. Following the rest, the cells were restimulated with soluble anti-CD3 0.5 μg/ml, goat anti-hamster 20 μg/ml (Vector Labs, Burlingame, CA) with or without stimulatory 2 μg/ml anti-ICOS (Clone: C398.4A, eBioscience). To determine whether ICOS signaling inactivated, we collected FOXO1 cells at 30 or 24 hr post restimulation and analyzed FOXO1-GFP compared to DRAQ5 staining using AMNIS ImageStream and BD LSR Fortessa analysis. To determine whether ICOS signaling through FOXO1 might be involved in ICOS upregulation of BCL6, we left WT or Foxo1TKO CD4 T cells in culture for 24 hr post restimulation and analyzed expression of Tfh markers by flow cytometry.

Generation, Infection, and Analysis of Mixed Bone-Marrow Chimeras

Bone-marrow cells were harvested from femurs, tibias, and humeri. T cells were depleted from bone-marrow cell suspensions with anti-Thy1.2 (30-H12, eBioscience) and low-toxicity rabbit complement (Cedarlane Laboratories). CD45.1+ wild-type bone-marrow cells were mixed with 4-fold excess CD45.2+ Foxo1TKO bone-marrow cells. 5–10^6 total bone-marrow cells were infected into lethally irradiated (10 Gy) CD45.1.2+ hosts. Eight weeks later, chimerism was assessed by flow cytometry and mice were injected intravenously with 10^6 ActA-Lm-OVA i.v. (Ertelt et al., 2009). Nine days later, mice were sacrificed, spleen and lymph node cells were harvested, and lymphocytes were stained for 1 hr at room temperature with LLO:p-A:A- streptavidin-allophycocyanin tetramers and 2 mg of phycoerythrin-conjugated antibody specific for CXCR5 (2G8; Becker Dickinson). Samples were then enriched for bead-bound cells on magnetized columns (Moon et al., 2007). Cells were then analyzed by flow cytometry.

Statistical Analyses

Unless otherwise indicated two-tailed, unpaired Student t tests were used to determine statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2015.01.017.
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