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OX40 Costimulation Inhibits Foxp3 Expression and Treg Induction via BATF3-Dependent and Independent Mechanisms

Xiaolong Zhang[1], **Xiang Xiao**[1], **Peixiang Lan**[1], **Junhui Li**[1], **Yaling Dou**[1], **Wenhao Chen**[1], **Naoto Ishii**[2], **Shuqiu Chen**[1], **Bo Xia**[3], **Kaifu Chen**[3], **Elizabeth Taparowsky**[4], and **Xian C. Li**[1,5,6,*]

[1]Immunobiology and Transplant Science Center and Department of Surgery, Houston Methodist Hospital, Texas Medical Center, Houston, TX, USA

[2]Department of Microbiology and Immunology, Tohoku University, Sendai, Japan

[3]Department of Cardiovascular Sciences, Houston Methodist Research Institute, Houston, TX, USA

[4]Department of Biological Sciences, Perdue University, Lafayette, IN, USA

[5]Department of Surgery, Weill Cornell Medical College of Cornell University, New York, NY, USA

[6]Lead Contact

SUMMARY

Naive $CD4^+$ T cells can be converted to $Foxp3^+$ T regulatory cells (Tregs) in the periphery (iTregs), where induction of *Foxp3* gene expression is central to Treg differentiation. OX40 signaling is known to inhibit *Foxp3* expression and Treg induction, but the underlying mechanisms remain poorly defined. Here, we found that OX40 costimulation activates two distinct molecular pathways to suppress *Foxp3* expression in freshly activated naive $CD4^+$ T cells. Specifically, OX40 upregulates BATF3 and BATF, which produce a closed chromatin configuration to repress Foxp3 expression in a Sirt1/7-dependent manner. Moreover, OX40 can also activate the AKT-mTOR pathway, especially in the absence of BATF3 and BATF, to inhibit Foxp3 induction, and this is mediated by phos-phorylation and nuclear exclusion of the transcription factor Foxo1. Taken together, our results provide key mechanistic insights into how OX40 inhibits Foxp3 expression and Treg induction in the periphery.

*Correspondence: xcli@houstonmethodist.org. **AUTHOR CONTRIBUTIONS** X.Z., X.X., P.L., J.L., and Y.D. designed and performed experiments; W.C. and E.T. provided helpful discussions; and S.C. contributed to animal breeding and handling. E.T. and N.I. provided the *Batf3*-Tg and OX40L-Tg mice. B.X. and K.C. provided ATAC-seq analysis. X.Z. and X.C.L. supervised the studies and wrote the paper.

DATA AND SOFTWARE AVAILABILITY

The accession numbers for the microarray and ATAC-seq data reported in this paper are GEO: GSE104611 and GSE114568.

Data and Software Availability

GSE104611

GSE114568

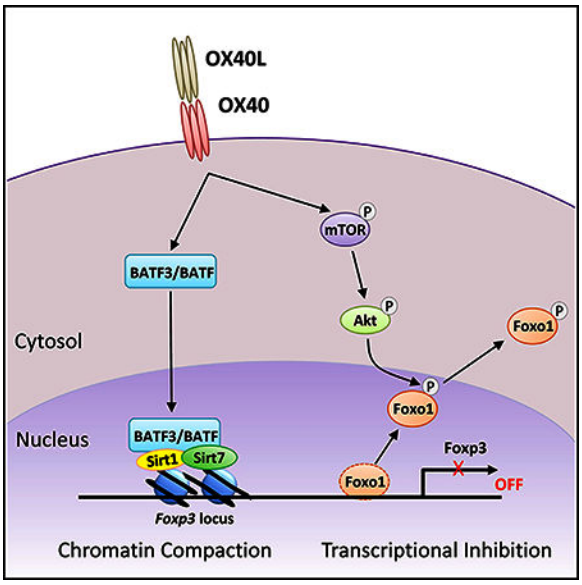
SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one table and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.06.052.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Graphical Abstract



In Brief

Zhang et al. show that OX40 inhibits Foxp3 expression by upregulating BATF and BATF3 expression in activating CD4$^+$ T cells, and BATF proteins close the Foxp3 locus by recruiting the histone deacetylases Sirt1/7. Additionally, OX40 activates the AKT-mTOR pathway to inhibit Foxp3 expression in the absence of the BATF proteins.

INTRODUCTION

CD4$^+$Foxp3$^+$ T regulatory cells (Tregs) play a critical role in immune homeostasis and peripheral tolerance (Sakaguchi et al., 2008). Their development, lineage stability, and suppressor functions are dependent on the expression of the transcription factor Foxp3, which is a ‘‘master’’ regulator of Treg identity (Hori et al., 2003). In addition to their thymic origin (natural Tregs ornTregs), CD4$^+$Foxp3$^+$ Treg cells can also be induced in the periphery from naive CD4$^+$ T cells following activation, which are often called inducible Tregs (iTregs) or peripheral Tregs (pTregs) (Curotto de Lafaille and Lafaille, 2009). The best characterized conditions for the induction of iTregs *in vitro* is the combination of transforming growth factor β (TGF-β) and interleukin-2 (IL-2); these cytokines potently induce *de novo* Foxp3 expression, which programs the conversion of activated conventional T cells to iTregs (Chen et al., 2003; Zheng et al., 2007). It has been shown that, besides Foxp3, multiple other transcription factors (e.g., Smad3, Foxo1, Foxo3, STAT5, AP-1, RUNX, nuclear factor KB [NF-KB], and E2A) are also involved in Foxp3 expression and iTreg cell differentiation, and these transcription factors are controlled by TCR signaling, cytokines, as well as signals for the T cell costimulatory molecules, thus highlighting the complexity of Foxp3 expression and Treg induction in the periphery (Tone and Greene, 2011).

OX40 is a T cell costimulatory molecule that belongs to the tumor necrosis factor receptor (TNFR) superfamily. Unlike other costimulatory molecules, OX40 is not expressed by resting naive T cells, but its expression is rapidly induced upon T cell activation, and OX40 costimulation plays a critical role in cell survival, proliferation, and generation of memory cells (Croft, 2010). We and others have shown that OX40 regulates the induction of multiple T helper (T_H) cell subsets, primarily through triggering chromatin modifications that control accessibility of key transcription factors to target loci (Xiao et al., 2016, 2018). In certain models, OX40 strongly promotes the induction of T_H1, T_H2, and T_H9 cells, whereas in other conditions, it inhibits the generation of T_H17 cells and $Foxp3^+$ Tregs (Ito et al., 2005; Li et al., 2008; Vu et al., 2007; Xiao et al., 2012a). However, the exact mechanisms by which OX40 inhibits Foxp3 gene expression and consequently iTreg cell induction in the periphery remain poorly defined. OX40 traditionally signals through the NF-kB pathway, and under certain conditions, OX40 also triggers the activation of NFAT, mitogen-activated protein kinase (MAPK), and AKT/mTOR pathways (So et al., 2011; Xiao et al., 2012b). Some of those pathways clearly overlap with those downstream of T cell receptor (TCR) stimulation. But how such signaling pathways interact with each other and then integrate in activated $CD4^+$ T cells, resulting in suppression of Foxp3 expression, is an issue of considerable importance.

In the present study, we addressed this issue by profiling transcriptional changes in naive $CD4^+$ T cells activated under iTreg-polarizing conditions with or without OX40 engagement and identified BATF3 and BATF as potent repressors of Foxp3 expression. The BATF proteins belong to theAP-1 family of transcription factors (Dorsey et al., 1995; Iacobelli et al., 2000). We found that BATF3 and BATF are strongly induced by OX40 costimulation; they bind to the *Foxp3* locus and inhibit Foxp3 expression by recruiting the histone deacetylases Sirt1 and Sirt7. We also found that, in the absence of both BATF3 and BATF, OX40 costimulation activates the AKT-mTOR pathway to suppress Foxp3 expression and iTreg induction, thus demonstrating the potential importance of OX40 in suppressing Treg induction in the periphery.

RESULTS

OX40 Costimulation, BATF3 Induction, and Inhibition of Foxp3 Expression

Following standard protocols for iTreg induction (Zheng et al., 2007), we fluorescence-activated cell sorting (FACS) sorted naive $CD4^+$ T cells ($CD62L^+CD44^{low}$ $Foxp3^-$) from wild-type (WT) *Foxp3*- EGFP reporter mice and activated them *in vitro* with anti-CD3/ antigen-presenting cells (APCs) in the presence of TGF-β and IL-2. To deliver OX40 costimulation to activated T cells, we used OX40L-Tg APCs in the culture. As shown in Figure 1A, TGF-β and IL-2 converted a substantial fraction of naive $CD4^+$ T cells to $Foxp3^+$ T cells (~60%) 3 days after the culture, and this conversion was completely inhibited by OX40 and very few naive $CD4^+$ T cells became $Foxp3^+$ cells in the presence of OX40 costimulation (~2%), which is consistent with our previous reports (Xiao et al., 2012a). We examined the iTregs in the gut of WT and OX40L-Tg mice, using Helios and Nrp1 as iTreg markers (Figure S1A; Thornton et al., 2010; Yadav et al., 2012). Indeed, iTregs in the gut lamina propria (LP), as defined by $CD4^+Foxp3^+$ but negative for both Helios and Nrp1 (i.e.,

$CD4^+Foxp3^+Helios^-Nrp1^-$), were markedly reduced in OX40L-Tg mice in both frequencies and absolute cell numbers as compared to those in WT B6 mice. Furthermore, we adoptively transferred naive $CD4^+CD25^-$ T cells from WT B6 or $OX40^{-/-}$ mice into Rag1 $^{-/-}$ or Rag1 $^{-/-}$ OX40L-Tg mice to directly investigate *in vivo* iTreg differentiation. We found that *in vivo* iTreg differentiation from the adoptively transferred naive WT, but not $OX40^{-/-}$, $CD4^+$ T cells was significantly reduced in OX40L-Tg mice (Figure S1B). This striking difference prompted us to perform Affymetrix gene array analysis, comparing changes in transcriptional profiles in activated $CD4^+$ T cells with or without OX40 costimulation. Among the genes that showed the most prominent changes in OX40-stimulated $CD4^+$ T cells, based on fold changes in gene expression, we plotted a cohort of 47 genes that exhibited the most striking differences between the two groups (Figure 1B). Among a cohort of genes that encode transcription factors, *Batf3* and *Batf* were among the highly upregulated genes in OX40-stimulated cells (Figure 1B). Using real-time qPCR analysis, we further confirmed the effect of OX40 costimulation in upregulation of *Batf3* and *Batf* in activated $CD4^+$ T cells. As shown in Figure 1C, as compared to the controls, expression of both *Batf3* and *Batf* genes was strongly upregulated in OX40-stimulated $CD4^+$ T cells in a time-dependent manner. We also performed assay for transposase accessible chromatin sequencing (ATAC-seq) experiments, assessing the global chromatin accessibility of *Foxp3* locus in $CD4^+$ T cells activated with or without OX40 costimulation and observed that, under iTreg-inducing conditions, the *Foxp3* locus, especially the CNS2 region, is highly accessible in $CD4^+$ T cells 2 days after activation (WT control, without OX40), which is completely inhibited upon OX40 costimulation (Figure 1D). As compared to $CD4^+$ T cells activated without OX40 costimulation, those activated with OX40 showed higher levels of open chromatin accessibility at the *Batf3* locus (Figure 1D), which is consistent with the increased BATF3 expression in OX40-stimulated T cells (Figure 1C). We also assessed the accessibility of the *Icos* locus, which is not related to OX40 as a control, and observed no marked differences in chromatin structure in activated $CD4^+$ T cells regardless of OX40 costimulation (Figure 1D).

BATF3 and BATF Are Potent Repressors of Foxp3 Expression

BATF3 and BATF are members of the AP-1 family transcription factors (Murphy et al., 2013). As shown in Figure 2A, sequence analysis of *Foxp3* locus revealed five putative AP-1 binding sites, which are located in the promoter, CNS1, and CNS2 regions. Their exact locations are depicted in relation to the transcription start site (TSS). To determine whether BATF3 is capable of binding to these sites, we introduced a Flag tag to BATF3 so that the BATF3 protein can be immunoprecipitated using an anti-Flag monoclonal antibody (mAb) (no anti-BATF3 Abs available now). We FACS sorted naive $CD4^+$ T cells from WT B6 mice and activated them with plate-bounded anti-CD3 and soluble anti-CD28 for 18 hr, followed by transducing activated $CD4^+$ cells with the BATF3-Flag-expressing retroviral vector or with a control vector. The transduced T cells were cultured in the presence of TGF-β and IL-2 for 48 hr, and chromatin immunoprecipitation (ChIP) analysis was performed to determine the enrichment of BATF3 at the *Foxp3* locus. As shown in Figure 2A, as compared to the control vector transduced cells, BATF3 was highly enriched at all putative AP-1 sites at the *Foxp3* locus (Figure 2A, bottom panel). Similar findings were observed for

the enrichment of BATF at the *Foxp3* locus (data not shown). Thus, these data demonstrate that BATF3 and BATF can physically bind to the *Foxp3* locus.

To determine whether BATF3 and BATF are functionally involved in suppressing Foxp3 expression and iTreg induction, we again overexpressed BATF3 in activated $CD4^+$ T using the retroviral mediated gene transfer approach and examined the induction of $Foxp3^+$ T cells by TGF-β and IL-2 *in vitro.* In these experiments, the transduced T cells were marked by the expression of GFP, which allows the analysis of Foxp3 expression in successfully transduced T cells under iTreg-polarizing conditions. We found that, in BATF3 transduced $CD4^+$ T cells, Foxp3 expression and induction of $Foxp3^+$ T cells were strongly inhibited. As compared to control-vector-transduced $CD4^+$ T cells, where ~50% of the GFP^+ cells became $Foxp3^+$ T cells, only ~15% BATF3-transduced $CD4^+$ T cells were $Foxp3^+$ T cells under the same culture conditions (Figures 2B and S2A). In a different set of experiments, we FACS sorted naive $CD4^+$ T cells from WT B6 and *Batf3-Tg* mice (both carrying the *Foxp3-GFP* reporter gene) and assessed their ability to convert to $Foxp3^+$ T cells under the iTreg-culturing conditions. As shown in Figure 2C, although a significant fraction of WT $CD4^+$ T cells were converted into $Foxp3^+$ T cells by TGF-β and IL-2 (~60%), only a small fraction of *Batf3-Tg* $CD4^+$ T cells became $Foxp3^+$ T cells (~10%), suggesting that the *Batf3-Tg* $CD4^+$ T cells are highly resistant to the induction of $Foxp3^+$ iTregs. Of note, naive $CD4^+$ T cells from WT B6 control mice and *Batf3-Tg* mice showed similar profiles of proliferation following activation *in vitro* (Figure 2D). Furthermore, overexpression of BATF in activated $CD4^+$ T cells also suppressed the induction of Foxp3 in a similar fashion as that of BATF3 (Figures 2E and S2B). We performed co-culture experiments involving WT B6 and *Batf3-Tg* $CD4^+Foxp3^-$ T cells, which are segregated by congenic markers (CD45.1 versus CD45.2), and examined Foxp3 induction under identical conditions. We found that the Foxp3 expression is suppressed in *Batf3-Tg* CD4 T cells, but not in WT CD4 T cells (Figure S2C), excluding the possibility of cytokines in suppression of Foxp3 expression. We also assessed the iTreg in *Batf3-Tg* mice and found that *Batf3-Tg* mice had reduced number of iTreg cells in the gut as compared to that in WT B6 mice (Figure S3). Together, these data suggest that BATF3 and BATF are potent repressors of Foxp3 expression.

BATF3 Expression Induces Extensive Chromatin Remodeling at the *Foxp3* Locus

Induction of Foxp3 expression requires the cytokines TGF-β and IL-2, which signal through the Smad proteins and STAT5, respectively (Burchill et al., 2007; Tone et al., 2008). To determine whether BATF3 overexpression would affect the cytokine signaling, we FACS sorted naive $CD4^+$ T cells from WT B6 and *Batf3-Tg* mice and activated them with anti-CD3/APCs in the presence of TGF-β and IL-2. At different time points after activation (0–72 hr), we analyzed the nuclear accumulation of phos-phorylated Smad3 and STAT5 in $CD4^+$ T cells. As shown in Figures 3A and 3B, phosphorylation of Smad3 and STAT5 in the nuclear extracts was comparable between WT and *Batf3*-Tg $CD4^+$ T cells at all time points examined. Thus, suppression of Foxp3 expression and iTreg induction by BATF3 is unlikely due to altered cytokine signaling events.

We next examined the chromatin modification status at the *Foxp3* locus, focusing specifically on the acetylation of H3 and H4 in activated WT and Batf3-Tg $CD4^+$ T cells (an

open chromatin status). We activated naive CD4$^+$ T cells from WT B6 and *Batf3*-tg mice with anti-CD3/APCs, with or without TGF-β and IL-2 for 48 hr, and then performed ChIP assays using specific mAbs against acetylated histone proteins. As shown in Figure 3C, activation of WT B6 CD4$^+$ T cells under iTreg conditions resulted in strong acetylation of H3 and H4 at the *Foxp3* promoter region, as well as the CNS1 and CNS2 regions. Strikingly, in the activated *Batf3-Tg* CD4$^+$ T cells, the H3 and H4 acetylation was inhibited. Similarly, H3K9 and H3K27 were hyper-acetylated in activated WT B6 CD4$^+$ T cells but strongly inhibited in activated *Batf3-Tg* CD4$^+$ T cells (Figure 3D). Of note, activation of either WT CD4$^+$ T cells or *Batf3*-Tg CD4$^+$ T cells under non-polarizing conditions (in the absence of TGF-β and IL-2) did not show marked histone acetylation at the *Foxp3* locus (Figures 3C and 3D). These data suggest that BATF3 is capable of mediating chromatin remodeling that inhibits *Foxp3* gene expression.

BATF3 Recruits the Histone Deacetylases Sirt1 and Sirt7 to *Foxp3* Locus to Suppress Foxp3 Gene Expression

BATF3 is a transcription factor without known deacetylase activities (Hildner et al., 2008; Iacobelli et al., 2000). We reasoned that BATF3 may modify the acetylation status of *Foxp3* locus by recruiting histone deacetylases (HDACs and Sirt proteins). To test this possibility, we overexpressed the Flag-tagged BATF3 in activated WT CD4$^+$ T cells using the retroviral mediated gene transfer approach and examined whether BATF3 could interact with key histone deacetylases in co-immunoprecipitation assays. We cultured the retroviral transduced CD4$^+$ T cells under iTreg-polarizing conditions for 48 hr, immunoprecipitated BATF3 with the anti-Flag mAb (no available anti-BATF3 mAb for immunoprecipitation), followed by immunoblotting for known HDACs and Sirt proteins. As shown in Figure 4A, none of the HDACs examined in the current study (HDAC1–7) co-immunoprecipitated with BATF3 in the activated CD4$^+$ T cells. However, Sirtl and Sirt7 proteins were readily detected in the BATF3 immunoprecipitates, suggesting that BATF3 may bind and recruit Sirtl and Sirt7 to mediate chromatin deacetylation at the *Foxp3* locus.

We took the following approaches to assess the role of BATF3 and Sirt1/7 in suppression of Foxp3 expression and iTreg induction. First, we activated WT B6 and *Batf3-tg* CD4$^+$ T cells under the iTreg conditions and added protein deacetylase inhibitors trichostatin A (TSA) or sodium butyrate (NaB) in some of the cultures. Induction of Foxp3 expression in activated CD4$^+$ T cells was examined 3 days later. NaB is a broad protein deacetylase inhibitor, inhibiting most protein deacetylases, including the Sirt family deacetylases (class III family; Davie, 2003; Yoo et al., 2015). As shown in Figure 4B, inclusion of NaB in the cultures markedly rescued Foxp3 from BATF3-mediated suppression, as compared to cultures with DMSO controls (60% versus ~5%). However, TSA primarily inhibits HDACs activities (class I, II, and IV families), but not Sirt activities (Vanhaecke et al., 2004), and addition of TSA in the cultures showed minimal effects in rescuing iTregs from BATF3-mediated suppression (Figure 4B). Second, we used the short hairpin RNA (shRNA) approach to selectively knock down Sirt1 and Sirt7 in activated *Batf3-Tg* CD4$^+$ T cells and examined the induction of Foxp3 by TGF-β and IL-2. As shown in Figures 4C, S4A, and S4B, knockdown of both Sirt1 and Sirt7 in *Batf3-Tg* CD4$^+$ T cells had the most effects in rescuing Foxp3 induction than knockdown of either one alone. Figures S4A and S4B confirmed the

knockdown efficiency of Sirt1 and Sirt7 by shRNAs in comparison to control shRNAs. We also showed that knockdown of Sirt1 or Sirt7 had no significant effects in Foxp3 induction in activated *Batf3*$^{-/-}$*Batf*$^{-/-}$ CD4^{+} T cells (Figure S4C). Thus, the Sirt1 and Sirt7 protein deacetylases appear to be particularly important in BATF3-mediated suppression of Foxp3 expression.

OX40 Remains Capable of Suppressing Foxp3 Expression in the Absence of BATF3 and BATF

To test whether BATF3 is necessary and sufficient in OX40-mediated suppression of Foxp3, we activated WT B6 and *Batf3*$^{-/-}$ CD4^{+} T cells under iTreg-inducing conditions with or without OX40 costimulation and then examined the induction of Foxp3 3 days later. As shown in Figure 5A, WT CD4^{+} T cells was readily converted to Foxp3^{+} T cells by TGF-β and IL-2 (~55%), which was strongly inhibited by OX40 costimulation (~3%). Surprisingly, OX40 costimulation strongly inhibited the induction of Foxp3, as well as the conversion of *Batf3*$^{-/-}$ CD4^{+} T cells to Foxp3^{+} T cells. We consistently observed that OX40 costimulation had similar effects in suppression of Foxp3 induction from either WT B6 or *Batf3*$^{-/-}$ T cells (Figure 5A). One possibility is that BATF3 and BATF proteins may be redundant in that BATF may compensate for the deficiency of BATF3 (Tussiwand et al., 2012), as both are capable of suppressing the induction of Foxp3 expression (Figure 2). To test this possibility, we FACS sorted CD4^{+} T cells from *Batf*$^{-/-}$ mice and examined the role of OX40 in suppression of Foxp3 expression. We found that, similar to WT B6 CD4^{+} T cells, *Batf*$^{-1}$- T cells were readily converted to Foxp3^{+} T cells under iTreg-inducing conditions (~60%), and OX40 costimulation was extremely potent in suppression of Foxp3 expression and iTreg induction from *Batf*$^{-/-}$ CD4^{+} T cells (Figure 5B).

We then bred BATF3 and BATF double knockout (Batf3$^{-/-}$Batf$^{-/-}$) mice and further examined the role of BATF3 and BATF in OX40-mediated suppression of Foxp3 induction from CD4^{+} T cells deficient for both BATF3 and BATF. As shown in Figure 5C, similar to WT B6 CD4^{+} T cells, Batf3$^{-/-}$Batf$^{-/-}$ CD4^{+} T cells were readily converted to Foxp3^{+} T cells by TGF-β and IL-2 (~65%). Intriguingly, OX40 costimulation remained strongly suppressive in the induction of Foxp3 expression from Batf3$^{-/-}$Batf$^{-/-}$ CD4^{+} T cells. As compared to WT B6 T cells, more Batf3$^{-/-}$Batf$^{-/-}$ CD4^{+} T cells became Foxp3^{+} T cells in the presence of OX40 costimulation (6% versus 18%), but the suppression of Foxp3 expression remained very strong as compared to that without OX40 costimulation (~18% versus ~68%; Figure 5C). We also assessed the iTregs in the gut of Batf$^{-/-}$Batf3$^{-/-}$ mice and found that the relative frequencies and the absolute cells counts of Helios^{-} iTregs in Batf $^{-/-}$Batf3$^{-/-}$ mice were substantially increased as compared to those in WT B6 mice. Interestingly, the frequency of iTregs that do not express both Helios and Nrp1 (Helios and Nrp double-negative iTregs) appeared to be decreased (Figure S5). As BATF and BATF3 are involved in regulating a myriad of genes (Delacher et al., 2017; Hayatsu et al., 2017; Vasanthakumar et al., 2015), it is possible that in BATF3- and BATF-deficient iTregs, Nrp1 expression may be impacted, resulting in such discrepancies. We performed additional experiments addressing the role of sodium butyrate in Foxp3 induction in BATF and BATF3 double-deficient T cells. We did observe a small but a significant increase in Foxp3 expression in BATF and BATF3 double-deficient T cells treated with sodium butyrate

(Figure S6), which indicates that sodium butyrate may have other effects in Foxp3 induction, in addition to those triggered by the BATF proteins. Collectively, these data suggest that, despite their potency in suppression of *Foxp3* expression, BATF3 and BATF themselves do not fully account for the suppression of Foxp3 expression and iTreg induction by OX40.

Role of the AKT-mTOR Pathway in Suppression of Foxp3 Expression in BATF3 and BATF Double-Deficient T Cells

The inhibition of Foxp3 induction by OX40 in Batf3$^{-/-}$Batf$^{-/-}$ CD4^{+} T cells prompted us to examine other signaling pathways activated by OX40 in suppression of Foxp3. We FACS sorted naive CD4^{+} T cells from WT B6 and Batf$^{-/-}$Batf3$^{-/-}$ mice, activated them with anti-CD3 plus either WT B6 APCs or OX40L-Tg APCs, and then examined activation of the AKT-mTOR pathway at different time points by immunoblot analysis. As shown in Figure 6A, in either WT or Batf3$^{-/-}$Batf$^{-/-}$ CD4^{+} T cells, OX40 costimulation resulted in AKT and mTOR activation, as revealed by the enhanced phosphorylation status of AKT and mTOR. We also observed strong Foxo1 phosphorylation, especially in the nucleus of OX40-stimulated T cells, which is in sharp contrast to CD4^{+} T cells activated without OX40 costimulation. The quantitation data, as well as statistical analysis of the western blot experiments, were shown in Figure S7A.

As the AKT-mTOR pathway is known to affect Foxp3 induction, primarily through phosphorylation and nuclear exclusion of Foxo1/3 (Merkenschlager and von Boehmer, 2010; Ouyang et al., 2010), we examined to what extent OX40 uses this pathway to suppress Foxp3 expression and iTreg induction. We sorted naive CD4^{+} T cells from WT B6 mice and activated them with anti-CD3/OX40L-Tg APCs under iTreg-polarizing conditions. In some cultures, we added the mTOR inhibitor rapamycin, and induction of Foxp3^{+} T cells was examined 3 days later. As shown in Figure 6B, as compared to the controls (without rapamycin), addition of rapamycin partially rescued the OX40-mediated suppression of Foxp3 induction (~3% versus ~35%). In the cultures where CD4^{+} T cells were activated without OX40, addition of rapamycin further enhanced the expression of Foxp3 as well as the induction of Foxp3^{+} T cells (~80%). We further confirmed these results by using a phosphatidylinositol 3-kinase (PI3K) inhibitor, which also inhibits the AKT-mTOR pathway (Figure S7B). Interestingly, when naive CD4^{+} T cells from Batf$^{-/-}$Batf3$^{-/-}$ mice were activated in the presence of OX40 costimulation, rapamycin completely abolished the effects of OX40 in suppression of Foxp3 induction (Figure 6C). As compared to OX40-stimulated Batf3$^{-/-}$Batf$^{-/-}$ CD4^{+} T cells without rapamycin (~17%) or OX40-stimulated WT B6 CD4^{+} T cells in the presence of rapamycin (~35%), Foxp3 expression was induced at high levels from OX40-stimulated Batf$^{-/-}$Batf3$^{-/-}$ CD4^{+} T cells in the presence of rapamycin (~70%; Figure 6C). These results suggest that, in BATF3- and BATF-deficient T cells, OX40 primarily activates the AKT-mTOR pathway to suppress Foxp3 expression and iTreg induction.

We adoptively transferred a population of naive WT B6 and Batf$^{-/-}$Batf3$^{-/-}$ CD4^{+} T cells (FACS sorted CD4^{+}Foxp3^{-} cells; CD45.2^{+}) into CD45.1^{+} WT B6 (control groups) or CD45.1 $^{+}$ OX40L-Tg mice; some host mice were treated with rapamycin for 4 days post-cell transfer and then examined the induction of Foxp3^{+} T cells within the CD45.2^{+} fraction 7

days after the cell transfer. In control groups, induction of Foxp3+ T cells from WT B6 or *Batf*$^{-/-}$*Batf3*$^{-/-}$ CD4^{+}T cells were similar in either the host lymph nodes or in the lungs (Figure 7A). But in CD45.1^{+} OX40L-Tg mice groups, a distinct population of Foxp3^{+} T cells was induced from *Batf3*$^{-/-}$*Batf*$^{-/-}$ CD4^{+} T cells in either the host lymph nodes or in the lungs, and treatment of the host mice with rapamycin further enhanced the induction of Foxp3^{+} T cells. In contrast, induction of Foxp3^{+} T cells from WT B6 CD4^{+} T cells in OX40L-Tg hosts was insignificant (Figures 7B and 7C).

DISCUSSION

OX40 costimulation is known to suppress the induction of Foxp3^{+} Tregs from naive CD4^{+} T cells in the periphery, but the underlying mechanisms remain poorly defined. In the present study, we have demonstrated that OX40 costimulation activates two distinct molecular pathways in freshly activated naive CD4^{+} T cells that are functionally involved in suppressing Foxp3 expression and iTreg induction. One involves the induction of BATF3 and BATF in activated CD4^{+} T cells by OX40, where they suppress Foxp3 expression by closing the *Foxp3* locus. The other involves the activation of AKT-mTOR pathway, which inhibits Foxp3 expression by phosphorylation and nuclear exclusion of Foxo1/3, transcription factors that are critical in Foxp3 expression (Merkenschlager and von Boehmer, 2010). We provide evidence that OX40 promotes BATF3 and BATF expression in activated T cells and that BATF3/BATF bound to the promoter, as well as the CNS1 and CNS2 regions of the *Foxp3* locus, and through recruitment of Sirt1/7, they produced a closed chromatin configuration at *Foxp3* locus. We also demonstrate that OX40 remains capable of suppressing Foxp3 expression, especially in the absence of BATF3 and BATF, where OX40 activates the AKT-mTOR pathway to suppress Foxp3 expression and iTreg induction. Thus, our data provide the mechanistic insights into the role of OX40 in suppression of Foxp3 expression and may have important clinical implications in targeting the OX40 pathway for therapeutic purposes.

BATF3 and BATF are members of the AP-1 family transcription factors. Traditionally, they dimerize with JunB and act as repressors of AP-1 activities in activated T cells (Echlin et al., 2000). Earlier studies demonstrated that BATF3 and BATF potently inhibit the IL-2 promoter activities in activated T cells. They competitively bind JunB, which prevents JunB from dimerizing with the Fos proteins to form the AP-1 complex (Iacobelli et al., 2000). In certain models, BATF3 also serves as a positive transcription factor, especially upon partnering with IRF4, in supporting the development of CD8a^{+} dendritic cells (DCs) (Hildner et al., 2008; Tussiwand et al., 2012). Recent studies also suggest that BATF3 and BATF also play an important role in mediating the differentiation of Th17 cells and Th9 cells (Jabeen et al., 2013; Schraml et al., 2009). In this study, we demonstrate additional mechanisms by which BATF proteins regulate target gene expression. Clearly, BATF3 and BATF can physically bind to chromatin modifiers, such as the histone deacetylases Sirtl and Sirt7, as shown in co-immunoprecipitation (colP) experiments, and by recruiting such chromatin modifiers to the *Foxp3* locus, BATF proteins then mediate extensive chromatin modifications that profoundly affect *Foxp3* gene expression. Indeed, in multiple settings where BATF3 and BATF are expressed at high levels, induction of Foxp3 expression, and consequently Foxp3^{+} Tregs from activated T cells, is suppressed, and this Foxp3 suppression

is due, to a large extent, to a closed chromatin configuration at the *Foxp3* locus. These findings reveal a mechanism for the function of BATF proteins in the control of not only gene expression but also cell fate decisions.

The *Foxp3* gene transcription is regulated by a constellation of transcription factors involving complex mechanisms. Transcription factors that can bind the regulatory regions of *Foxp3* locus include Smad3, STAT5, NFAT, CREB, ATF, Foxo1, Foxo3, E2A, RUNX3, and c-Rel; most of them act as positive regulators of *Foxp3* transcription, whereas few of them (e.g., GATA-3 and Stat3) are negative regulators in suppressing Foxp3 expression (Tone and Greene, 2011). On the other hand, the epigenomic mechanisms that are involved in modifying the *Foxp3* locus are less well defined. But this is an emerging area with considerable importance; the current belief is that robust chromatin remodeling precedes the induction of transcriptional activities, including in the generation of Foxp3$^+$ Tregs (Arvey et al., 2014). Rudensky et al. (Zheng et al., 2010) reported that hyper-acetylation of his tones H3 and H4 in the promoter and CNS regions of *Foxp3* locus is critically important in the induction of Foxp3$^+$ Tregs. Also, the suppressive functions of Foxp3$^+$ Tregs can be profoundly affected by HDAC inhibitors (de Zoeten et al., 2010; Tao et al., 2007). Hancock et al. (Wang et al., 2015) reported that deficiency of HDAC3 in Foxp3$^+$ Tregs led to impaired Treg functions, with the subsequent development of autoimmunity, as well as a failure to induce tolerance to allografts. We provide compelling evidence that the cell surface costimulatory receptor OX40 is also a powerful regulator of Treg induction in the periphery. It is remarkable that OX40 costimulation triggers the activation of multiple signaling pathways that converge on the suppression of Foxp3 expression. It has been well known that OX40 also inhibits the suppressive functions of natural Tregs (Piconese et al., 2008; Vu et al., 2007). Collectively, these data highlight the importance of OX40 in counter-balancing the immune suppression exerted by Foxp3$^+$ Tregs, and although this situation may be desirable in cancer immuno-therapy (Hirschhorn-Cymerman et al., 2009; Piconese et al., 2008), compromising Treg functions can also result in autoimmunity and loss of immune homeostasis (Xiao et al., 2012b).

The finding that OX40 activates different signaling pathways in suppression of Foxp3$^+$ Tregs is highly interesting. BATF3 and BATF, which are strongly induced by OX40, are potent repressors of *Foxp3* expression, as overexpression of BATF proteins in activated T cells, either through retroviral mediated gene transfer or using *Batf3-Tg* T cells, strongly inhibited Foxp3 expression. However, BATF3 and BATF are not sufficient in OX40-mediated suppression of Foxp3 expression, as deficiency of both BATF3 and BATF in activated T cells fails to prevent OX40 from suppressing Foxp3 expression. Interestingly, the complete rescue of Foxp3 expression from OX40-mediated suppression in *Batf3*$^{-/-}$*Batf*$^{-/-}$ CD4$^+$ T cells treated with rapamycin suggests a critical role of the mTOR pathway as an alternative mechanism in suppression of Tregs by OX40. This notion was further supported by our analysis of AKT-mTOR activation in *Batf3*$^{-/-}$*Batf*$^{-/-}$ CD4$^+$ T cells activated in the presence of OX40 costimulation. However, how such two pathways interact in suppression of Foxp3 expression by OX40, and subsequently in Treg induction, remains to be defined. Clearly, OX40 is remarkably efficient in modifying the *Foxp3* locus, as shown in the ATAC-seq data. Moreover, overexpression of BATF proteins strongly inhibited Foxp3 induction in WT CD4$^+$ T cells, but inhibition of the AKT-mTOR pathway only partially rescued Foxp3 induction in

the presence of OX40 costimulation. Nevertheless, such two pathways inhibit Foxp3 induction via very different mechanisms. As mentioned above, BATF3 and BATF inhibit Foxp3 expression through chromatin remodeling, whereas activation of the AKT-mTOR-AKT pathway inhibits Foxp3 expression via phosphorylation of Foxo1 (Merkenschlager and von Boehmer, 2010; Ouyang et al., 2010). The relative significance of individual pathways in the control of tolerance versus immunity *in vivo* also warrants further investigation.

In summary, we have uncovered the mechanistic insights into how OX40 inhibits the expression of Foxp3 and induction of iTreg cells. Our findings emphasize the importance of OX40 in regulation of Foxp3$^+$ Tregs in a way that OX40 activates multiple signaling pathways in activated CD4$^+$ T cells that converge on the suppression of Foxp3 expression. Specifically, BATF3 and BATF inhibit Foxp3 expression through epigenomic mechanisms, and the AKT-mTOR pathway represses Foxp3 expression via the Foxo1 transcription factor. These findings provide insights into the molecular mechanisms that control Foxp3$^+$ Tregs by OX40 and may have important clinical implications.

EXPERIMENTAL PROCEDURES

Further details are in the Supplemental Experimental Procedures.

Animals

C57BL/6 (B6), BALB/c, *Batf3*$^{-/-}$, and *Batf*$^{-/-}$ mice were obtained from the Jackson Laboratory (Bar Harbor, ME). TheFoxp3-EGFP reporter mice and OX40L-Tg mice have been previously described (Xiao et al., 2012b). *Batf3* transgenic *(Batf3-Tg)* mice were kindly provided by Dr. E.J. Taparowsky. Batf3$^{-/-}$Baff$^{-/-}$ double knockout mice were generated by crossing the *Batf3*$^{-/-}$ and Baf$^{-/-}$ mice, and the double-deficient genotype was selected by PCR-assisted geno-typing. *Batf3*-Tg mice were crossed with *Foxp3*-GFP to get *Batf3*-Tg-*Foxp3*-GFP mice. 8-week-old male mice were used in the experiments. All animal care and experiments were approved by the Institutional Animal Care and Use Committee at the Houston Methodist Research Institute in Houston, Texas.

Microarray Analysis

For RNA preparation, naive CD4$^+$ T cells (CD62L^{high}CD44^{low}Foxp3$^-$) were sorted from *Foxp3-EGFP* reporter mice and activated *in vitro* with soluble anti-CD3 mAb and mitomycin-C-treated WT APCs or OX40L-Tg APCs in 96-well round-bottom tissue-culture plates. Forty-eight hours later, living CD4$^+$ T cells were sorted from cultures by flow cytometry. Total RNA was extracted with RNeasy mini kit (QIAGEN). cDNAs were then synthesized using Superscript III kits (Invitrogen); hybridization of the samples with Affymetrix mouse arrays was then performed at the Harvard Medical School Facility according to the manufacturer's protocol. The procedures for microarray data analyses were described previously by Swanson et al. (2009).

ATAC-Seq

ATAC-seq was performed as previously described (Buenrostro et al., 2013). Briefly, naive CD4$^+$ T cells were FACS sorted from *Foxp3-GFP* reporter mice with FACSaria (BD

Biosciences). The sorted naive $CD4^+$ T cells (1×10^5 cells/well) were stimulated with anti-CD3 plus equal numbers of WT APCs or OX40L-Tg APC in the presence of TGF-β1 (3 ng/mL; R&D Systems) and IL-2 (10 ng/mL; R&D Systems) for 48 hr. $CD4^+$ T cells were harvested after the culture and resuspended in 500 μL of growth media plus 5% DMSO and then transferred to a 1.7-mL microfuge tube on ice. The cells were frozen at a slow cooling rate. ATAC-seq was performed at Active Motif (Carlsbad, CA). ATAC-seq peaks were detected with DANPOS2 deregion function (Chen et al., 2015) and visualized using University of California, Santa Cruz (UCSC) genome browser with peak height normalized against the *Gapdh* locus. Gain and loss regions are defined by height q value < 0.01 and log2 fold change > 2. Associated genes are selected based on region from upstream 3 kb to downstream 10 kb of gene promoter. Pathway enrichment analysis was performed using DAVID (Huang da et al., 2009).

ChIP assay

ChIP assays were performed as previously described (Xiao et al., 2015). All primers used in ChIP-PCR are displayed in Table S1. Anti-H3Ac (39139), anti-H4Ac (39243), anti-H3K27Ac (39133; all from Active Motif), anti-H3K9Ac (ab4441; Abcam), and anti-Flag (F1804; Sigma-Aldrich) were used for the immunoprecipitation of chromatin with an EZ ChIP kit according to the manufacturer's instructions (Millipore). The precipitated DNA was then analyzed by real-time PCR. Data are presented as relative binding based on normalization to input DNA (Staudt et al., 2010).

Induction of $Foxp3^+$ iTregs *In Vivo*

FACS sorted naive $CD4^+Foxp3^-$ T cells (12×10^6 cells/mouse) from $CD45.2^+$ WT B6 or $CD45.2^+$ $Baff3^{-/-}Baff^{-/-}$ mice were adoptively transferred into $CD45.1^+$ WT or OX40L-Tg mice. The recipient mice were also injected with BALB/c splenic cells (10×10^6/mouse) and treated without or with rapamycin (3 mg/kg; intraperitoneally [i.p.]) on days 0, 1,3, and 5 post-cell transfer. Mice were sacrificed on day 7, and single-cell suspensions were prepared from the lymph nodes (LNs) and lung. Cells were stained with phycoerythrin (PE)-CY7-anti-CD4, APC-anti-CD45.1, and Alex Fluor 700-anti-CD45.2 mAbs, followed by intracellular staining of Foxp3 and flow cytometric analysis.

Statistical Analysis

Data were represented as mean ± SD and analyzed with Prism version 6.04 (GraphPad Software). Statistical analyses were performed using unpaired Student's t test. Differences were considered significant when $p < 0.05$. p values are denoted in figures as follows: ns, $p > 0.05$; *$p < 0.05$; and **$p < 0.01$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- OX40 costimulation upregulates BATF3 and BATF expression in activated $CD4^+$ T cells
- BATF proteins are potent repressors of Foxp3 expression
- Suppression function of BATF proteins is dependent on histone deacetylases Sirt1/7
- OX40 also activates the AKT-mTOR pathway to suppress Foxp3 expression

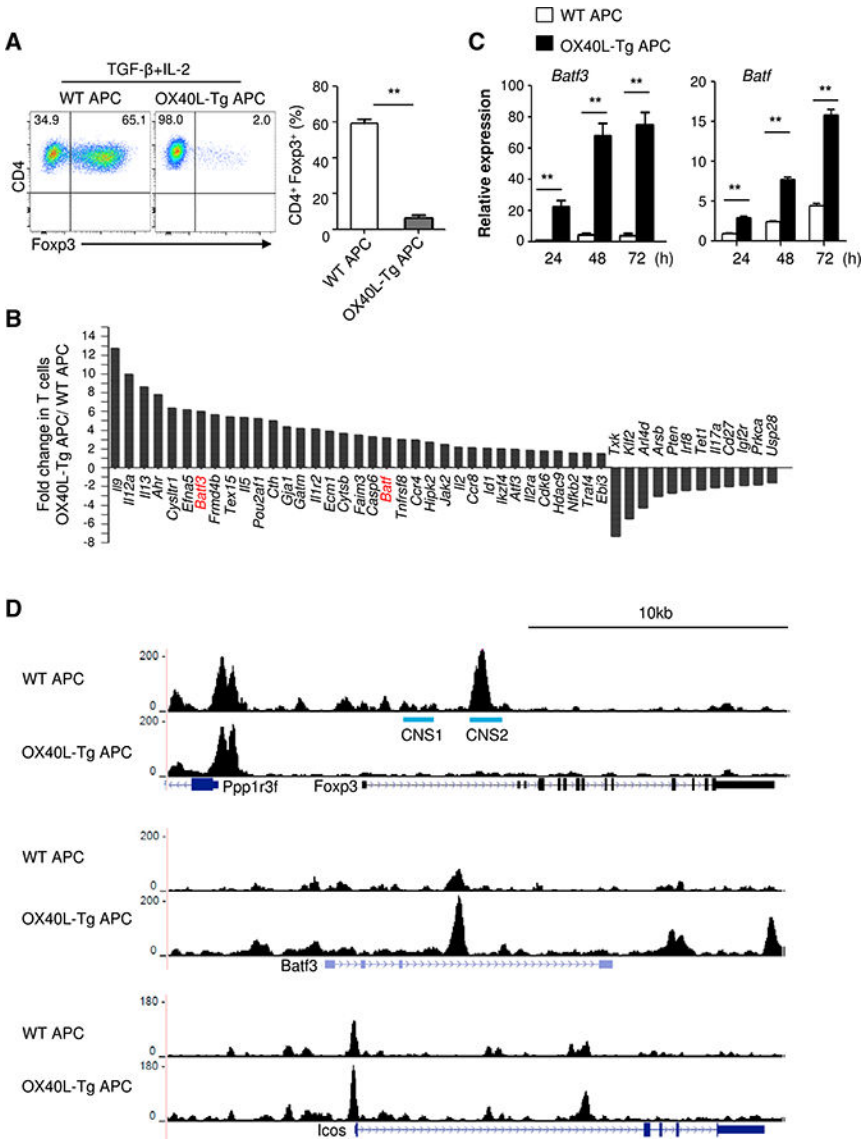

Figure 1. OX40 in Suppression of iTregs and Expression of the Transcription Factors BATF3 and BATF

(A) Naive$CD4^+Foxp3^-$ T cells were stimulated with WT or OX40L-Tg APCs under the iTreg-polarizing conditions, and induction of $Foxp3^+$ T cells among total $CD4^+$ T cells was shown 3 days later.

(B) Naive $CD4^+Foxp3^-$ T cells were stimulated with anti-CD3 plus WT or OX40L-Tg APCs under non-polarizing condition for 48 hr. RNA was obtained for Affymetrix array analysis. The bar graph shows the genes that were upregulated or down-regulated >2 folds in OX40L-Tg versus WT APCs stimulated T cells.

(C) *Batf3* and *Batf* mRNA expression in $CD4^+$ T cells at indicated hours post-polarization. Data represent mean values ± SD (n = 3).

(D) Naive $CD4^+$ T cells (1×10^5 cells/well) were stimulated with anti-CD3 plus WT APCs or OX40L-Tg APC in the presence ofTGF-β1 and IL-2 for 48 hr. Cells were obtained for ATAC-seq, peak height normalized against the *Gapdh* locus.

**p < 0.01.

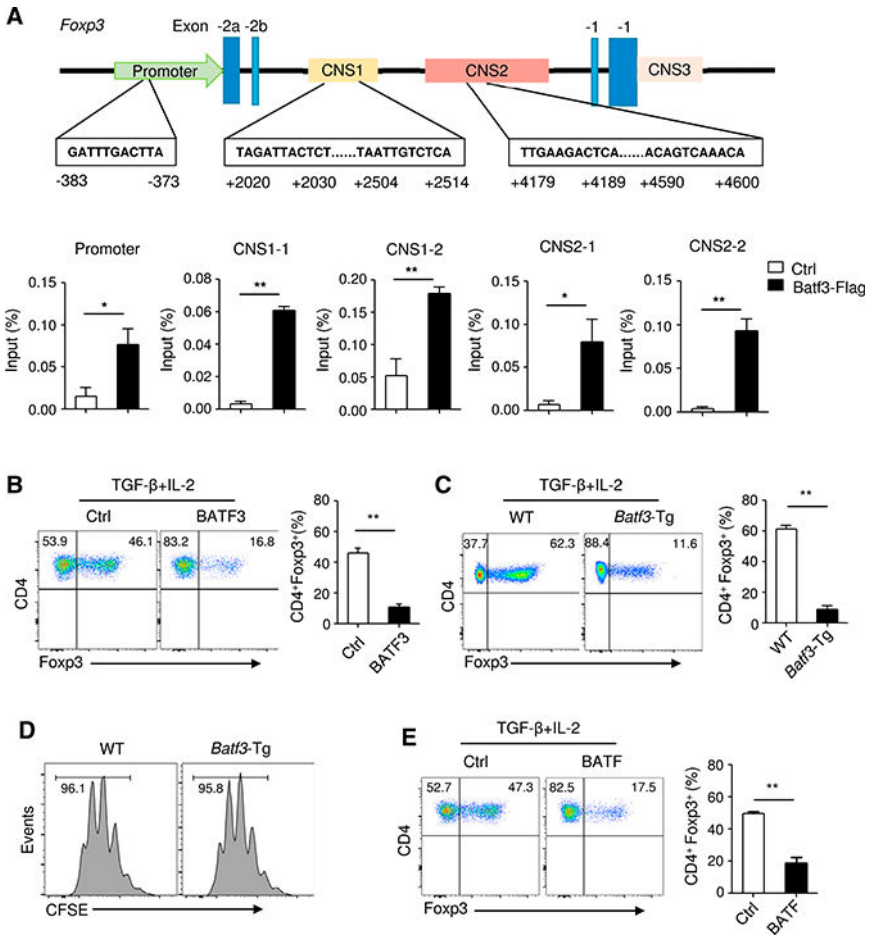

Figure 2. BATF3 and BATF Are Potent Repressors of Foxp3 Expression

(A) The graph on top illustrates the putative Jun binding sites (boxed) in the *Foxp3* promoter and the CNS1 and CNS2 regions. The bar graphs show the ChIP analysis of BATF3 enrichment at these Jun binding sites in $CD4^+$ T cells, which were transduced with an empty retroviral vector (Ctrl vector) or retrovirus expressing of BATF3 (*Batf3*-Flag vector) and then cultured under the iTreg-polarizing condition for 48 hr.

(B) $CD4^+CD25^-$ T cells from WT C57BL/6 mice were transduced with empty retroviral vector (GFP-Ctrl) or retrovirus expressing of BATF3 (BATF3-GFP) and cultured under the iTreg-polarizing condition for 2 days, followed by flow cytometry analysis. (%) $Foxp3^+$ cells in retrovirus transduced GFP^+ CD4 T cells were shown.

(C and D) Naive $CD4^+Foxp3^-$ T cells were sorted from WT and *Batf3-Tg* Foxp3-EGFP reporter mice and activated under iTreg-polarizing condition for 3 days.

(C) (%) $Foxp3^+$ cells in CD4 T cells.

(D) WT and *Batf3-Tg* $CD4^+Foxp3^-$ T cells were stained with carboxyfluroescein succinimidyl ester (CFSE).

(E) $CD4^+CD25^-$ T cells from WT B6 mice were transduced with empty retroviral vector (GFP-Ctrl) or retrovirus expressing of BATF (BATF-GFP) and followed analysis as in (B).

Data (A, B, C, and E) represent mean values ± SD (n = 3). $^{**}p < 0.01$.

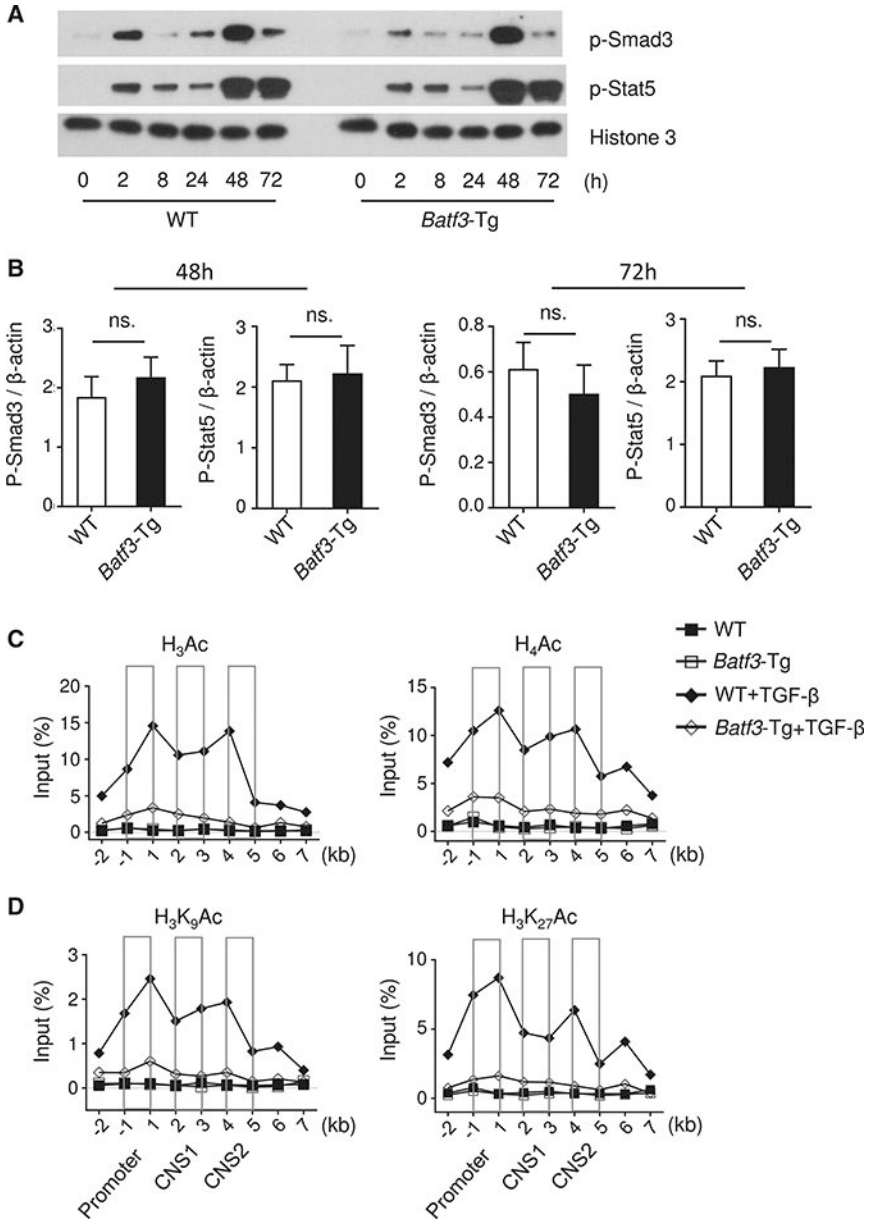

Figure 3. BATF3 Inhibits Histone Acetylation at the *Foxp3* Locus under iTreg-Polarizing Conditions

(A) Immunoblot analysis of phosphorylated Smad3 and Stat5 in the nucleus of naive WT or *Batf3-Tg* $CD4^+$ T cells at indicated hours upon iTreg polarization.

(B) Quantitation as well as statistical analysis of the Smad3 and Stat5 expression in western blot experiments.

(C and D) FACS sorted naive $CD4^+Foxp3^-$ T cells (C and D) from WT or *Batf3*-Tg *Foxp3*-EGFP reporter mice were cultured under the iTreg-polarizing condition (with TGF-β and IL-2) or nonpolarizing condition conditions (without TGF-β and IL-2) for 48 hr. Shown are ChIP analysis of H3Ac, H4Ac (C), H3K9Ac, and H3K27Ac (D) at *Foxp3* locus.

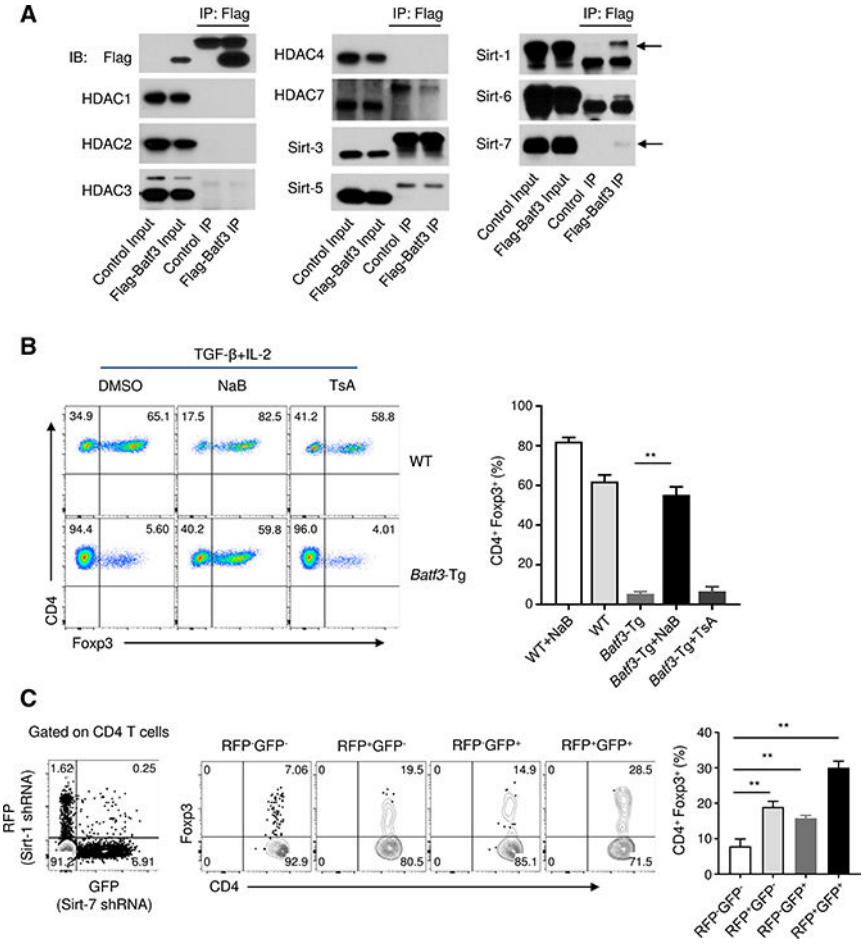

Figure 4. BATF3 Inhibits Histone Acetylation by Recruiting the Histone Deacetylases Sirt1 and Sirt7 to the *Foxp3* Locus

(A) $CD4^+$ Tcells were transduced with BATF3-Flag and cultured under the iTreg-polarizing conditions for 48 hr, followed by co-immunoprecipitation analysis of BATF3 (Flag). Anti-Flag immunoprecipitates (IPs) were subjected to immunoblot analysis (IB) by anti-Sirt and anti-HDAC antibodies.

(B) Flow cytometry analysis of Foxp3 expression in WT and *Batf3-Tg* $CD4^+$ T cellsthat were activated under the iTreg-polarizing conditions for 3 days in the presence of pan-HDACs inhibitor NaB (250 μg/mL) or TSA (1 nM).

(C) $CD4^+CD25^-$ T cells from Batf3-Tg mice were transduced with retrovirus expressing of Sirt1 shRNA and Sirt7 shRNA and cultured under the iTreg-polarizing condition for 2 days, followed by flow cytometry analysis. (%) $Foxp3^+$ cells in retrovirus transduced CD4 T cells were shown.

Data (B and C) represent mean values ± SD(n = 3).

**p<0.01.

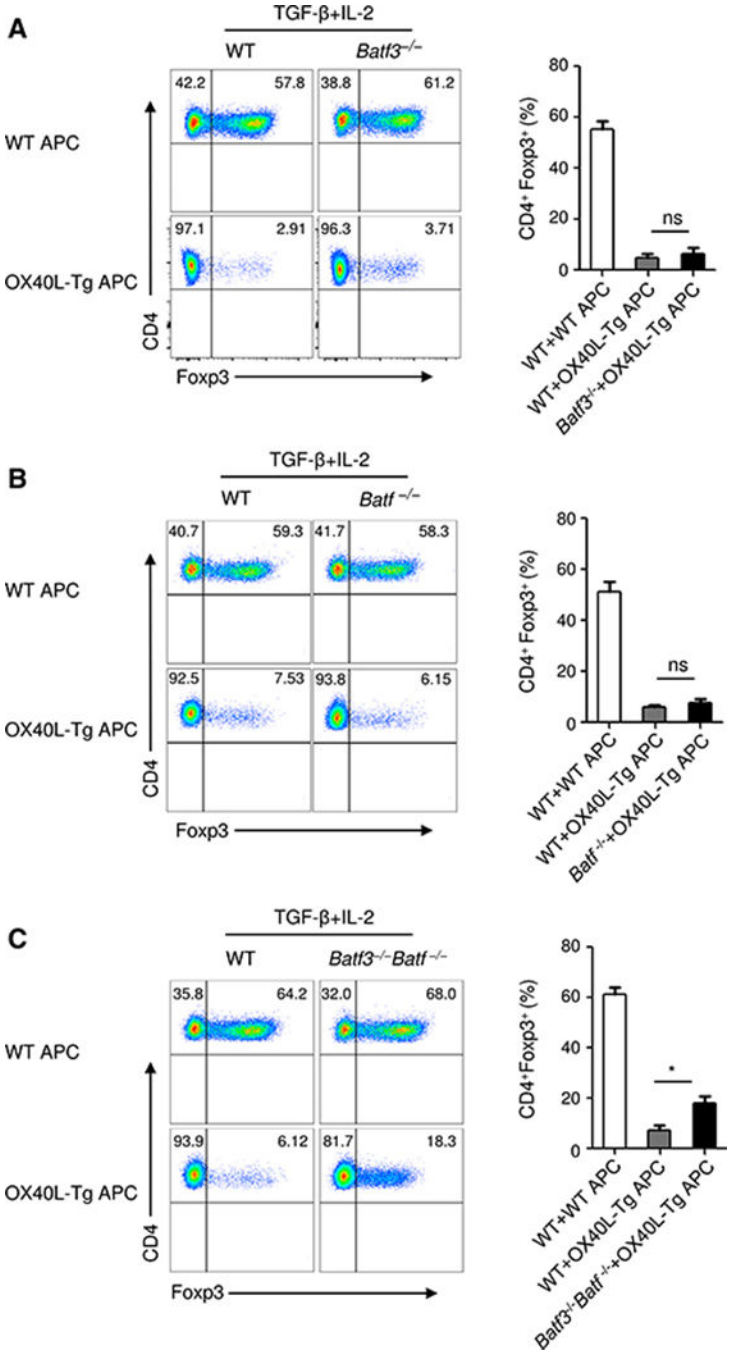

Figure 5. OX40 Strongly Inhibits iTreg Induction in BATF3- and BATF-Deficient CD4$^+$ T Cells

Naive CD4$^+$ T cells were sorted from WT, *Batf3*$^{-/-}$, *Batf*$^{-/-}$, and *Batf*$^{-/-}$*Batf3*$^{-/-}$ mice, stimulated with WT or OX40L-Tg APCs, and cultured under the iTreg-polarizing conditions for 3 days, followed by flow cytometry analysis.

(A) (%) Foxp3$^+$ cells in WT and *Batf3*$^{-/-}$ CD4 T cells.

(B) (%) Foxp3$^+$ cells in WT and *Batf*$^{-/-}$ CD4 T cells.

(C) (%) Foxp3$^+$ cells in WT and *Batf*$^{-/-}$*Batf3*$^{-/-}$ CD4 T cells. *$p < 0.05$.

Data (A-C) represent mean values ± SD (n = 3).

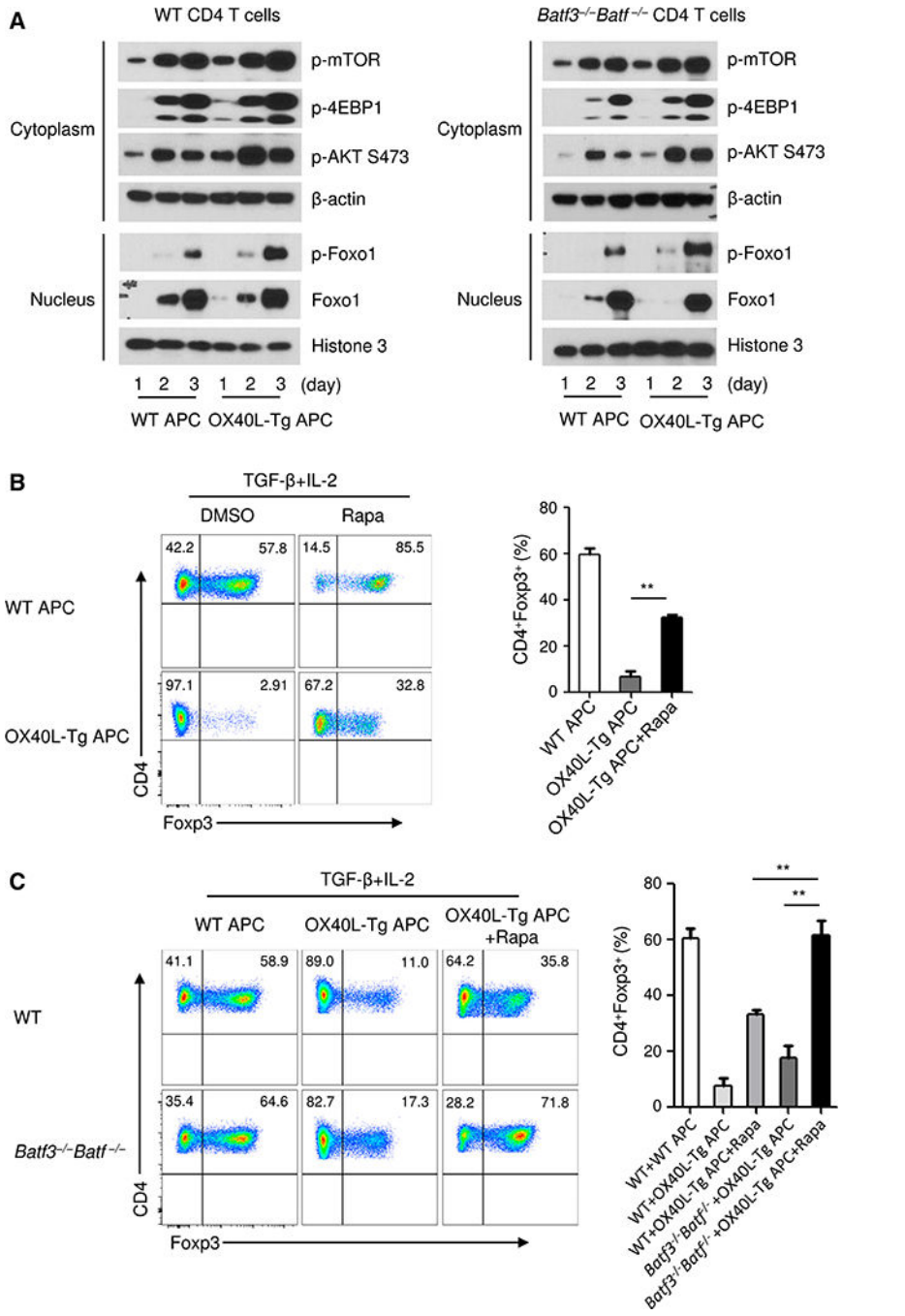

Figure 6. OX40 Activates AKT-mTOR Pathway to Suppress iTreg Induction in BATF3- and BATF-Deficient CD4$^+$ T Cells

(A) Naive CD4$^+$CD25$^-$ T cells were sorted from WT and *Batf*$^{-/-}$*Batf3*$^{-/-}$ mice, stimulated with WT or OX40L-Tg APCs, and cultured under the iTreg-polarizing condition. Shown are immunoblot analyses of phosphorylated mTOR, AKT, and 4EBP1 in the cytosol and total Foxol and phosphorylated Foxol in the nucleus at indicated days post-polarization.

(B) Naive CD4$^+$ T cells from WT mice stimulated with WT or OX40L-Tg APCs and cultured under the iTreg-polarizing conditions supplemented with rapamycin (25 nM) or DMSO. Plots and the bar graph show the %Foxp3$^+$ cells in cultured CD4$^+$ T cells.

(C) Naive $CD4^+$ T cells from WT and *Batf*$^{-/-}$*Batf3*$^{-/-}$ mice were activated and cultured as described in (B). Plots and the bar graph show the %$Foxp3^+$ cells in cultured $CD4^+$ T cells. Data (B and C) represent mean values ± SD (n = 3).

**p < 0.01.

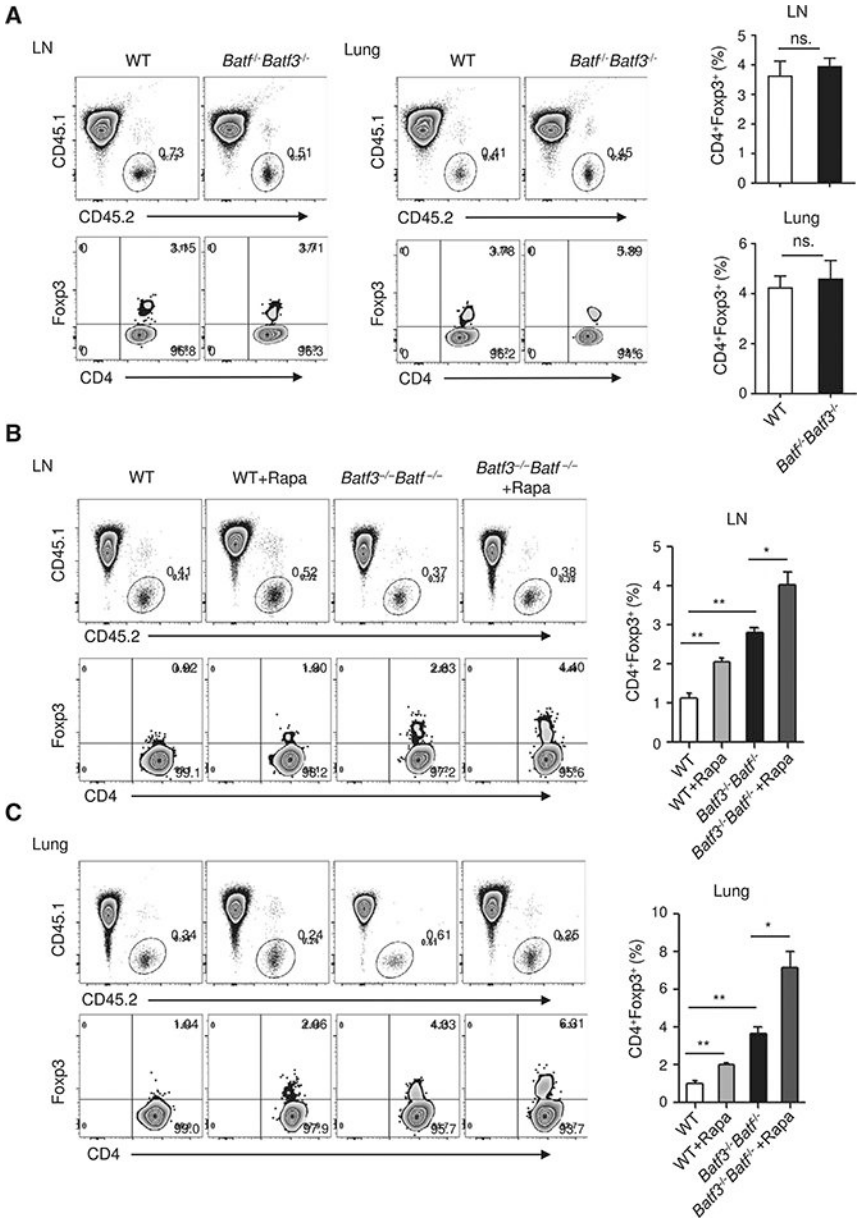

Figure 7. Role of mTOR Inhibition and BATF3 and BATF Deficiency in iTreg Induction in OX40L-Tg Hosts *In Vivo*

(A-C) CD45.1$^+$ WT B6 (A) or CD45.1$^+$ OX40L-Tg mice (B and C) were adoptively transferred with naive CD4$^+$Fxop3$^-$ T cells sorted from CD45.2$^+$ WT or Batf$^{-/-}$Batf3$^{-/-}$ mice and treated without or with rapamycin on days 0, 1,3, and 5. On day 7 post-cell transfer, leucocytes in mesenteric lymph nodes (LNs) and lungs were isolated for flow cytometry analysis. The dot plots and bar graphs indicate the (%) Foxp3$^+$ cells among the adoptively transferred CD45.2$^+$ cell populations. Data represent mean values ± SD (n = 3). *p < 0.05, **p < 0.01.