Published in final edited form as:

J Immunol. 2016 May 1; 196(9): 3537–3541. doi:10.4049/jimmunol.1501896.

Foxp1 controls naive CD8⁺ T cell quiescence by simultaneously repressing key pathways in cellular metabolism and cell cycle progression

Hairong Wei¹, Jianlin Geng¹, Bi Shi¹, Zhenghui Liu¹, Yin-Hu Wang¹, Anna C. Stevens¹, Stephanie L Sprout¹, Min Yao², Haikun Wang^{2,§}, and Hui Hu^{1,§}

¹Department of Microbiology, School of Medicine, University of Alabama at Birmingham, Birmingham, AL 35205

²Key Laboratory of Molecular Virology & Immunology, Institute Pasteur of Shanghai, Chinese Academy of Science, Shanghai 200031, China

Abstract

Previously we have shown that transcription factor Foxp1 plays an essential role in maintaining naive T cell quiescence; in the absence of Foxp1, mature naive CD8⁺ T cells proliferate in direct response to homeostatic cytokine IL-7. Here we report that the deletion of Foxp1 in naive CD8⁺ T cells leads to enhanced activation of PI3K/Akt/mTOR signaling pathway and its downstream cell growth and metabolism targets in response to IL-7. We found that Foxp1 directly regulates Pik3ip1, a negative regulator of PI3K. In addition, we found that deletion of Foxp1 in naive CD8⁺ T cells results in increased expression levels of *E2fs*, the critical components for cell cycle progression and proliferation, in a manner that is not associated with increased phosphorylation of retinoblastoma protein (Rb). Taken together, our studies suggest that Foxp1 enforces naive CD8⁺ T cell quiescence by simultaneously repressing key pathways in both cellular metabolism and cell cycle progression.

Introduction

Lymphocyte quiescence refers to a cellular state characterized by small cell size, low metabolic activity and lack of spontaneous proliferation. A considerable amount of literature has suggested that the quiescence of naive T cells in the periphery is not a default state: the survival of quiescent naive T cells requires constant sub-threshold signals from the engagement of the T cell antigen receptor (TCR) and stimulation of the IL-7 receptor (IL-7R) (1, 2), and multiple factors, including transcription factors have been found to actively maintain T cell quiescence (3-6). Previously we have shown that transcription factor Foxp1 plays a critical role not only in the generation of quiescent naive T cells during

[§]Address correspondence and reprint requests to Dr. Haikun Wang or Dr. Hui Hu, Key Laboratory of Molecular Virology & Immunology, Institute Pasteur of Shanghai, Chinese Academy of Science, Shanghai, China (H.W.); Department of Microbiology, School of Medicine, University of Alabama at Birmingham, Birmingham, AL (H.H.). hkwang@ips.ac.cn (H. W.), huihu@uab.edu (H. H.).

Disclosures

The authors have no financial conflicts of interest.

thymocyte development but also in the maintenance of quiescence of mature naive T cells in the periphery (7, 8). Compared with the other reported T cell quiescence genes (3-6), Foxp1 regulation of T cell quiescence shows a unique propensity in that the acute deletion of Foxp1 in mature naive CD8⁺ T cells allows the cells to proliferate in direct response to the stimulation of homeostatic cytokine IL-7 (8). The underlying mechanism, however, is still largely unknown.

When cells proliferate, cell metabolism and growth increase to both maintain the homeostatic control of cell size and support the cell division. In T lymphocytes, the PI3K/Akt/mTOR pathway is crucial for cell survival, growth and proliferation (9-11). Recently, PI3K interacting protein 1 (Pik3ip1) has been identified as a novel negative regulator that functions upstream of PI3K, leading to decreased Akt phosphorylation (12, 13). In addition to cell growth, cell cycle progression must also occur for cells to proliferate. Studies have shown that DNA replication and cell cycle progression is controlled by the tumor suppressor retinoblastoma protein (Rb)/E2F pathway (14-17). The sequential phosphorylation of Rb proteins by different cyclin-cdk complexes inactivates Rb, releasing E2F factors (E2F1, E2F2 and E2F3) from the repression E2F-Rb complexes (18-21). In T lymphocytes, it has been shown that E2F1 and E2F2 play important roles in maintaining T cell homeostasis; the deletion of both proteins diminishes lymphopenia-induced T cell proliferation (22).

In this study, we further investigated the underlying mechanism by which Foxp1 regulates naive T cell quiescence. We show that Foxp1 deletion in mature naive CD8⁺ T cells results in enhanced activation of PI3K/Akt/mTOR pathway, and Foxp1 regulates *Pik3ip1* directly. We also found that Foxp1-deficiency results in increased expression levels of *E2fs* and E2F1 indeed contributes to the proliferation of Foxp1-deficient naive CD8⁺ T cells in response to IL-7.

Materials and Methods

Mice

All mice were maintained in specific pathogen-free barrier facilities and were used in accordance with protocols approved by Institutional Animal Care and User Committee of the University of Alabama at Birmingham. *E2f1*^{-/-} mice were from Jackson Laboratories. *Foxp1*^{ΔTg}CD4-Cre and *Foxp1*^{f/f}Cre-ERT2⁺Rosa^{YFP} mice were generated as described (8). *Foxp1*^{f/f}Cre-ERT2⁺Rosa^{YFP} mice were crossed with *E2f1*^{-/-} mice to generate *E2f1*^{-/-}*Foxp1*^{f/f}Cre-ERT2⁺Rosa^{YFP} mice.

Flow cytometry and cell sorting

These procedures were done as described (8). All cell sorting was done on a FACS Aria (BD Biosciences). The sorted populations were >98% pure. Antibodies were as follows: Alexa Fluor 700-anti-CD44 (IM7, Biolegend), Brilliant Violet 785-anti-CD8α (53-6.7, Biolegend). Live/Dead Fixable Aqua Dead Cell fluorescence was from Invitrogen.

CellTrace Violet labeling and cell culture

A Violet Cell Proliferation Kit (Invitrogen) was used for analysis of cell proliferation. CD44^{lo}CD8⁺ T cells sorted from *Foxp1^{fl/f}Rosa^{YFP}*, *Foxp1^{fl/f}Cre-ERT2⁺Rosa^{YFP}*, *E2f1^{-/-}Foxp1^{fl/f}Rosa^{YFP}*, and *E2f1^{-/-}Foxp1^{fl/f}Cre-ERT2⁺Rosa^{YFP}* mice were labeled with CellTrace Violet following manufacturer's instructions. CellTrace Violet-labeled CD8⁺ T cells were cultured for 6 days with or without rmIL-7 (R&D systems) and 0.3 μ M 4-hydroxytamoxifen (Sigma). For chemical inhibition experiments, 10 μ M Ly294002 (PI3K inhibitor, Calbiochem) and/or 10 nM Rapamycin (mTOR inhibitor, MBL international) were added to the culture at day 0 and cell proliferation was analyzed at day 6. For enforced Pik3ip1 over-expression, Pik3ip1 was sub-cloned from pEF-PIK3IP1 (addgene, #49214) into the retroviral vector MSCV-IRES-Thy1.1 and retroviruses were produced as described (23). Cells were transduced with virus-containing medium supplemented with polybrene (6 μ g/ml) twice at day 4 by a plate centrifugation method. Akt phosphorylation and cell proliferation were analyzed at day 6.

Real-time reverse-transcription PCR

CD8⁺ T cells cultured with IL-7 were harvested at indicated time points. RNA was purified as described (8) for real-time PCR analysis of *Pik3ip1*, *E2f1*, *E2f2*, *E2f3*, *Cdk1*, *Mcm5* and *Pcna* mRNAs. The primers were provided in Supplemental Table 1.

Immunoblot analysis

CD8⁺ T cells cultured with IL-7 for a total of 4 days were harvested for immunoblot analysis. Cells were lysed and SDS-PAGE was done as described (8). Antibodies to phospho-S6 ribosomal protein (2F9), phospho-p70 S6 kinase (Ser371), Rb (D20), phospho-Rb (C84F6), and phospho-Akt (C31E5E) were from Cell signaling Technology. β -Actin (I-9) was from Santa Cruz. Pik3ip1 antibody (16826-1-AP) was from Proteintech.

ChIP

ChIP assay was done as described (8). Foxp1 precipitated DNA and input DNA were assessed by quantitative real-time PCR with Universal SYBR Green Supermix (Bio-Rad).

Statistical analysis

A two-tailed Student's t-test was used when two groups were compared for statistical differences. An ANOVA test was used when more than two groups were compared for statistical differences.

Results and Discussion

Foxp1-deficiency in naive CD8⁺ T cells leads to enhanced activation of PI3K/Akt/mTOR pathway in response to IL-7

To determine whether the PI3K/Akt/mTOR pathway plays a role in Foxp1-mediated quiescence regulation, we first used the pharmacological inhibitor blocking approach. As we have shown previously (8), naive YFP⁺ *Foxp1^{fl/f}Cre-ERT2⁺Rosa^{YFP}* (Foxp1-cKO) CD8⁺ T cells but not naive *Foxp1^{fl/f}Rosa^{YFP}* (Foxp1-WT) CD8⁺ T cells proliferated and increased

cell size in response to IL-7 *in vitro* (**Fig. 1A**). Interestingly, we found that Ly294002 and Rapamycin, the inhibitors of PI3K and mTOR, respectively, sufficiently abrogated both the proliferation and the increased cell size of Foxp1-cKO CD8⁺ T cells in response to IL-7 (**Fig. 1A**). We further examined the activation of Akt. In Foxp1-cKO CD8⁺ T cells cultured with IL-7 for a total of 4 days, a time point at which the cells had not proliferated but a significant fraction of the cells were in the S phase (data not shown), the phosphorylation of Akt was markedly enhanced compared to that in control Foxp1-WT CD8⁺ T cells (**Fig. 1B**). Furthermore, the phosphorylation of p70S6 kinase and its substrate ribosomal protein S6, was induced in Foxp1-cKO CD8⁺ T cells (**Fig. 1C**). Previously we have shown that Foxp1-deletion leads to elevated IL-7R expression (8). To determine whether enhanced Akt and p70S6 kinase activity in Foxp1-cKO CD8⁺ T cells is mainly caused by the elevated IL-7R expression, we cultured both Foxp1 WT and Foxp1-cKO CD8⁺ T cells with a high dosage of IL-7 (15 ng/ml) that nearly saturated the activation of IL-7R/Akt signaling (**Supplementary Fig. 1A**). We found that the phosphorylation of p70S6 kinase and S6 was induced only in Foxp1-cKO CD8⁺ T cells (**Supplementary Fig. 1B**), suggesting that the proliferation of Foxp1-deficient CD8⁺ T cells in response to IL-7 is not simply due to the elevated IL-7R; rather, there are also other Foxp1 targets involved in promoting the cell proliferation.

Taken together, these results suggest that Foxp1 deletion in naive CD8⁺ T cells results in increased PI3K/Akt/mTOR signaling in response to IL-7 and this pathway plays an important role in Foxp1-mediated regulation of T cell quiescence.

Foxp1 directly regulates the expression of *Pik3ip1*

Studies have shown that *Pik3ip1* is a negative regulator of PI3K (13). Interestingly, we found that in the cultures with IL-7, Foxp1-cKO CD8⁺ T cells expressed significantly lower levels of *Pik3ip1* protein compared with WT CD8⁺ T cells (**Fig. 2A**). Consistently, we found that Foxp1-cKO CD8⁺ T cells expressed significantly lower levels of *Pik3ip1* mRNA, while Foxp1A-transgene (Foxp1A Tg) enhanced its expression (**Fig. 2B**), suggesting that *Pik3ip1* could be a direct target of Foxp1. We performed the bioinformatics analysis and identified one forkhead-binding site with high scores in the promoter region of the *Pik3ip1* locus (**Fig. 2C**, left panel). Chromatin-immunoprecipitation (ChIP) assay of Foxp1 in mature wild-type CD8⁺ T cells showed that Foxp1 bound specifically to the *Pik3ip1* promoter region (**Fig. 2C**, right panel). To further address the function of *Pik3ip1*, we used retroviral expression approach and found that the over-expression of *Pik3ip1* in Foxp1-cKO CD8⁺ T cells reduced the Akt phosphorylation levels and the cell proliferation in response to IL-7 (**Fig. 2D**). As expected, the over-expression of Foxp1A in Foxp1-cKO CD8⁺ T cells also reduced the Akt phosphorylation levels and the cell proliferation in response to IL-7 (**Fig. 2D**). These results suggest that Foxp1 likely dampens PI3K/Akt/mTOR signaling via its direct control of *Pik3ip1* expression levels. Thus, Foxp1 enforces T cell quiescence by negatively regulating key pathways in cellular metabolism and cell growth.

Foxp1-deficiency in naive CD8⁺ T cells leads to induction of E2F factors

To determine how Foxp1-deficiency affects cell cycle progression, we first examined the phosphorylation of Rb. Rb proteins have multiple phosphorylation sites, and studies have shown that the Rb proteins phosphorylated at S780 cannot bind to E2F1 *in vivo* (18, 21). In

naive CD8⁺ T cells that were cultured with IL-7 for a total of 4 days, we found that the phosphorylation of Rb at S780 remained at basal levels in Foxp1-cKO CD8⁺ T cells as in control Foxp1-WT CD8⁺ T cells (**Fig. 3A**). We also did not find any differences at some other phosphorylation sites of Rb between Foxp1-WT and Foxp1-cKO CD8⁺ T cells (**Supplementary Fig. 1C**). Yet surprisingly, by day 4, the expression of *E2f1*, *E2f2* and *E2f3* mRNAs in Foxp1-cKO CD8⁺ T cells was induced to significantly higher levels than in Foxp1-WT CD8⁺ T cells at the IL-7 concentrations adequate enough to induce cell proliferation (**Figs. 3B and 4A**). Consistently, the expression levels of E2F targets *Cdk1* (24, 25), *Mcm5* (26), and *Pcna* (24, 25), which are all crucial for cell cycle progression, were also induced to higher levels in Foxp1-cKO CD8⁺ T cells (**Fig. 3C**). The expression of *E2fs* and their target genes was also examined at early time points of the culture before the cells entered the cell cycle. We found that the mRNAs of *E2f1*, *E2f3*, *Cdk1*, *Mcm5* and *Pcna*, but not *E2f2*, were already induced to higher levels in Foxp1-cKO than in Foxp1-WT CD8⁺ T cells (**Supplementary Fig. 1D and 1E**). The results suggest that in response to IL-7, the increased expression levels of cell cycle-related genes in Foxp1-deleted CD8⁺ T cells are directly due to Foxp1 deficiency, and are not associated with increased phosphorylation of Rb. It is interesting to note that at a relatively low IL-7 concentration of 0.6 ng/ml, *Cdk1* mRNA levels were increased in Foxp1-cKO CD8⁺ T cells in the absence of the induction of *E2fs* (**Fig. 3C**). The increased expression levels of *E2f1-3* at two higher IL-7 concentrations helped boost the *Cdk1* levels even higher in Foxp1-cKO CD8⁺ T cells (**Fig. 3C**). These results suggest that Foxp1 regulates important pathways involved in cell cycle progression.

E2F1 plays an important role in regulating T lymphocyte homeostasis (22). Early studies have shown that E2F1 stimulates its own transcription and both *E2f2* and *E2f3* are direct targets of E2F1 (27, 28). To determine whether the increased levels of *E2fs* contribute to the cell proliferation of Foxp1-deficient CD8⁺ T cells in response to IL-7, we generated *E2f1*^{-/-}*Foxp1*^{fl/fl}Cre-ERT2⁺*Rosa*^{YFP} mice. We found that Foxp1-cKO CD8⁺ T cells proliferated extensively in response to IL-7 by day 6 *in vitro*, whereas naive CD8⁺ T cells deficient of both *E2f1* and *Foxp1* (DKO) proliferated much less (**Fig. 4A**), suggesting that increased *E2f1* expression levels in Foxp1-cKO CD8⁺ T cells contributes to the proliferation of Foxp1-deficient CD8⁺ T cells in response to IL-7. However, the deletion of *E2f1* in Foxp1-cKO CD8⁺ T cells did not completely abrogate cell proliferation (**Fig. 4A**). We found that in the absence of *E2f1*, the expression levels of *E2f2* and *E2f3* were still induced to higher levels in Foxp1-cKO CD8⁺ T cells than in Foxp1-WT CD8⁺ T cells (**Fig. 4B**), suggesting that the increased levels of *E2f2* and *E2f3* likely compensate for the *E2f1* deficiency and contribute to the residual cell proliferation of DKO CD8⁺ T cells in response to IL-7. Taken together, our results suggest that via its repression on the expression levels of E2F factors, Foxp1 enforces T cell quiescence by negatively regulating cell cycle progression.

In summary, we have shown that Foxp1 functions as a critical regulator of T cell quiescence by simultaneously repressing key pathways in both metabolism and cell cycle progression. Foxp1 directly regulates a negative regulator of PI3K, *Pik3ip1*, the over-expression of which reduces both the Akt phosphorylation and the cell proliferation in response to IL-7. Foxp1 also represses the expression levels of *E2f* factors to enforce T cells quiescence. This study

sheds new light on the mechanism underlying T cell quiescence and provides potential targets for manipulating T cell quiescence and activation to treat a number of diseases such as cancer and autoimmune disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Marion Spell and Enid F. Keyser for excellent technical help with flow cytometry.

This work was supported by the Knowledge Innovation Program (Y414P11212) from the Chinese Academy of Sciences and China Young 1000-Talent Program (H.W), the Alliance for Cell Gene Therapy Foundation (H.H), US National Institutes of Health AI095439 and AI103162 (H.H), UAB CFAR Vaccine Concept Grant (H.H), and UAB Center for AIDS Research (P30AI027767-26).

Abbreviations used in this article

Rb	retinoblastoma protein
Pik3ip1	PI3K interaction protein 1
ChIP	Chromatin-immunoprecipitation
RV	retroviral vector
PI	Propidium iodide

References

1. Tan JT, Dudl E, LeRoy E, Murray R, Sprent J, Weinberg KI, Surh CD. IL-7 is critical for homeostatic proliferation and survival of naive T cells. *Proc Natl Acad Sci U S A*. 2001; 98:8732–8737. [PubMed: 11447288]
2. Takada K, Jameson SC. Naive T cell homeostasis: from awareness of space to a sense of place. *Nat Rev Immunol*. 2009; 9:823–832. [PubMed: 19935802]
3. Kerdiles YM, Beisner DR, Tinoco R, Dejean AS, Castrillon DH, DePinho RA, Hedrick SM. Foxo1 links homing and survival of naive T cells by regulating L-selectin, CCR7 and interleukin 7 receptor. *Nature immunology*. 2009; 10:176–184. [PubMed: 19136962]
4. Buckley AF, Kuo CT, Leiden JM. Transcription factor LKLF is sufficient to program T cell quiescence via a c-Myc-dependent pathway. *Nature immunology*. 2001; 2:698–704. [PubMed: 11477405]
5. Hamilton SE, Jameson SC. CD8 T cell quiescence revisited. *Trends in immunology*. 2012; 33:224–230. [PubMed: 22361353]
6. Yang K, Neale G, Green DR, He W, Chi H. The tumor suppressor Tsc1 enforces quiescence of naive T cells to promote immune homeostasis and function. *Nature immunology*. 2011; 12:888–897. [PubMed: 21765414]
7. Feng X, Ippolito GC, Tian L, Wiehagen K, Oh S, Sambandam A, Willen J, Bunte RM, Maika SD, Harriss JV, Caton AJ, Bhandoola A, Tucker PW, Hu H. Foxp1 is an essential transcriptional regulator for the generation of quiescent naive T cells during thymocyte development. *Blood*. 2010; 115:510–518. [PubMed: 19965654]
8. Feng X, Wang H, Takata H, Day TJ, Willen J, Hu H. Transcription factor Foxp1 exerts essential cell-intrinsic regulation of the quiescence of naive T cells. *Nature immunology*. 2011; 12:544–550. [PubMed: 21532575]

9. Okkenhaug K. Signaling by the phosphoinositide 3-kinase family in immune cells. *Annual review of immunology*. 2013; 31:675–704.
10. Chi H. Regulation and function of mTOR signalling in T cell fate decisions. *Nature reviews. Immunology*. 2012; 12:325–338. [PubMed: 22517423]
11. Magnuson B, Ekim B, Fingar DC. Regulation and function of ribosomal protein S6 kinase (S6K) within mTOR signalling networks. *The Biochemical journal*. 2012; 441:1–21. [PubMed: 22168436]
12. DeFrances MC, Debelius DR, Cheng J, Kane LP. Inhibition of T-cell activation by PIK3IP1. *European journal of immunology*. 2012; 42:2754–2759. [PubMed: 22706993]
13. Zhu Z, He X, Johnson C, Stoops J, Eaker AE, Stoffer DS, Bell A, Zarnegar R, DeFrances MC. PI3K is negatively regulated by PIK3IP1, a novel p110 interacting protein. *Biochem Biophys Res Commun*. 2007; 358:66–72. [PubMed: 17475214]
14. Chellappan SP, Hiebert S, Mudryj M, Horowitz JM, Nevins JR. The E2F transcription factor is a cellular target for the RB protein. *Cell*. 1991; 65:1053–1061. [PubMed: 1828392]
15. Dyson N. The regulation of E2F by pRB-family proteins. *Genes & development*. 1998; 12:2245–2262. [PubMed: 9694791]
16. Chen HZ, Tsai SY, Leone G. Emerging roles of E2Fs in cancer: an exit from cell cycle control. *Nature reviews. Cancer*. 2009; 9:785–797. [PubMed: 19851314]
17. Nevins JR. The Rb/E2F pathway and cancer. *Hum Mol Genet*. 2001; 10:699–703. [PubMed: 11257102]
18. Kitagawa M, Higashi H, Jung HK, Suzuki-Takahashi I, Ikeda M, Tamai K, Kato J, Segawa K, Yoshida E, Nishimura S, Taya Y. The consensus motif for phosphorylation by cyclin D1-Cdk4 is different from that for phosphorylation by cyclin A/E-Cdk2. *Embo J*. 1996; 15:7060–7069. [PubMed: 9003781]
19. Knudsen ES, Wang JY. Differential regulation of retinoblastoma protein function by specific Cdk phosphorylation sites. *J Biol Chem*. 1996; 271:8313–8320. [PubMed: 8626527]
20. Harbour JW, Luo RX, Dei Santi A, Postigo AA, Dean DC. Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1. *Cell*. 1999; 98:859–869. [PubMed: 10499802]
21. Lundberg AS, Weinberg RA. Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-cdk complexes. *Mol Cell Biol*. 1998; 18:753–761. [PubMed: 9447971]
22. DeRyckere D, DeGregori J. E2F1 and E2F2 are differentially required for homeostasis-driven and antigen-induced T cell proliferation in vivo. *Journal of immunology*. 2005; 175:647–655.
23. Wang H, Geng J, Wen X, Bi E, Kossenkova AV, Wolf AI, Tas J, Choi YS, Takata H, Day TJ, Chang LY, Sprout SL, Becker EK, Willen J, Tian L, Wang X, Xiao C, Jiang P, Crotty S, Victora GD, Showe LC, Tucker HO, Erikson J, Hu H. The transcription factor Foxp1 is a critical negative regulator of the differentiation of follicular helper T cells. *Nature immunology*. 2014; 15:667–675. [PubMed: 24859450]
24. Ren B, Cam H, Takahashi Y, Volkert T, Terragni J, Young RA, Dynlacht BD. E2F integrates cell cycle progression with DNA repair, replication, and G(2)/M checkpoints. *Genes & development*. 2002; 16:245–256. [PubMed: 11799067]
25. Zhu W, Giangrande PH, Nevins JR. E2Fs link the control of G1/S and G2/M transcription. *The EMBO journal*. 2004; 23:4615–4626. [PubMed: 15510213]
26. Ohtani K, Iwanaga R, Nakamura M, Ikeda M, Yabuta N, Tsuruga H, Nojima H. Cell growth-regulated expression of mammalian MCM5 and MCM6 genes mediated by the transcription factor E2F. *Oncogene*. 1999; 18:2299–2309. [PubMed: 10327050]
27. Bracken AP, Ciro M, Cocito A, Helin K. E2F target genes: unraveling the biology. *Trends Biochem Sci*. 2004; 29:409–417. [PubMed: 15362224]
28. Hsiao KM, McMahon SL, Farnham PJ. Multiple DNA elements are required for the growth regulation of the mouse E2F1 promoter. *Genes & development*. 1994; 8:1526–1537. [PubMed: 7958837]

FIGURE 1.

Foxp1-deletion in naive CD8⁺ T cells leads to enhanced activation of PI3K/AKT/mTOR pathway in response to IL-7. (A) Naive CD8⁺ T cells from *Foxp1^{fl/fl}Rosa^{YFP}* or *Foxp1^{fl/fl}Cre-ERT2⁺Rosa^{YFP}* mice were labeled with CellTrace Violet and cultured with tamoxifen and IL-7 (3 ng/ml) in the absence or presence of 10 μ M Ly294002 and/or 10 nM Rapamycin. At day 6, the proliferation and the size of the cultured cells were examined. (B, C) Naive CD8⁺ T cells from *Foxp1^{fl/fl}Rosa^{YFP}* or *Foxp1^{fl/fl}Cre-ERT2⁺Rosa^{YFP}* mice were cultured with tamoxifen and IL-7 (3 ng/ml) for 2 days. Foxp1-WT or Foxp1-cKO (YFP⁺) T cells were then sorted and cultured with IL-7 (3 ng/ml) for another 2 days; at day 4, the cultured cells were harvested for immunoblot analysis of phosphorylated (p-) Akt (B), p70S6K and S6 (C). β -Actin serves as a loading control. Data (A, B, C) are representative of three independent experiments.

FIGURE 2.

Foxp1 directly regulates the expression of *Pik3ip1*. **(A)** Naive CD8⁺ T cells from *Foxp1^{f/f}Rosa^{YFP}* or *Foxp1^{f/f}Cre-ERT2⁺Rosa^{YFP}* mice were cultured with tamoxifen and IL-7 (3 ng/ml) for 2 days, Foxp1-WT and Foxp1-cKO T cells were then sorted and cultured with IL-7 (3 ng/ml) for another 2 days before being harvested for immunoblot analysis of *Pik3ip1* protein. **(B)** Naive CD8⁺ T cells from *Foxp1^{f/f}Rosa^{YFP}*, *Foxp1^{f/f}Cre-ERT2⁺Rosa^{YFP}* or *Foxp1A^{Tg}TgCD4-Cre* mice were cultured with tamoxifen and IL-7 (3 ng/ml) for 2 days, Foxp1-WT, Foxp1-cKO and Foxp1A Tg T cells were then sorted and cultured with IL-7 (3 ng/ml) for another 2 days before being harvested for real-time PCR analysis of *Pik3ip1* mRNA; results are normalized to *Rpl32* mRNA and are presented relative to the *Pik3ip1* mRNA in Foxp1-WT CD8⁺ T cells. **(C)** Predicted forkhead-binding site (underlined) in the *Pik3ip1* promoter (left panel) and ChIP analysis of the binding of Foxp1 to a control region (Ctrl) or the promoter region (prom) of *Pik3ip1*, presented as binding in Foxp1-WT CD8⁺ T cells relative to binding in Foxp1-cKO CD8⁺ T cells (right panel). **(D)** Naive CD8⁺ T cells from *Foxp1^{f/f}Cre-ERT2⁺Rosa^{YFP}* mice were labeled with CellTrace Violet and cultured with tamoxifen and IL-7 (3 ng/ml). Cells were transduced with retroviruses at day 4; Akt phosphorylation and cell proliferation were analyzed by flow cytometry at day 6. Numbers indicate the mean fluorescence intensity (MFI) of staining. ** $P < 0.01$, *** $P < 0.001$. Data **(B, C)** are shown as mean $\pm$ SEM of three independent experiments. Data **(A, D)** are representative of two independent experiments.

FIGURE 3.

Foxp1-deletion leads to increased levels of *E2f1-3*. Naive CD8⁺ T cells from *Foxp1^{fl/f}Rosa^{YFP}* or *Foxp1^{fl/f}Cre-ERT2⁺Rosa^{YFP}* mice were cultured with tamoxifen and different concentrations of IL-7 for 2 days; Foxp1-WT or Foxp1-cKO T cells were then sorted and cultured with IL-7 (at the same concentrations as before) for another 2 days. At day 4, the cultured cells were harvested for: (A) immunoblot analysis of total Rb and phosphorylated Rb; (B, C) real-time PCR analysis of (B) *E2f1*, *E2f2* and *E2f3* mRNAs, and (C) *cdk1*, *mcm5* and *pcna* mRNAs in Foxp1-WT and Foxp1-cKO CD8⁺ T cells; results are normalized to *Rpl32* mRNA and are presented relative to the corresponding mRNAs in control Foxp1-WT CD8⁺ T cells. ***P*<0.01; ****P*<0.001. Data (A) are representative of three independent experiments; data (B, C) are shown as mean ± SEM of three independent experiments.

FIGURE 4.

E2F1 contributes to the cell proliferation of Foxp1-deficient naive CD8⁺ T cells in response to IL-7. Naive CD8⁺ T cells from *Foxp1^{f/f}Rosa^{YFP}*, *Foxp1^{f/f}Cre-ERT2⁺Rosa^{YFP}*, *E2f1^{-/-}Foxp1^{f/f}Rosa^{YFP}* or *E2f1^{-/-}Foxp1^{f/f}Cre-ERT2⁺Rosa^{YFP}* mice were (**A**) labeled with CellTrace violet and cultured with tamoxifen and different concentrations of IL-7 for 6 days before the cultured cells were analyzed for cell proliferation, or (**B**) cells were cultured with tamoxifen and IL-7 (3 ng/ml) for 2 days, Foxp1-WT, Foxp1-cKO, E2F1 KO, or E2F1/Foxp1 double-deficient (DKO) T cells were then sorted and cultured with IL-7 (3 ng/ml) for another 2 days. At day 4, the cultured cells were harvested for real-time PCR analysis of *E2f1*, *E2f2* and *E2f3* mRNAs; results are normalized to *Rpl32* mRNA and are presented relative to the corresponding mRNAs in Foxp1-WT CD8⁺ T cells. **P*<0.05; ***P*<0.01. Data (**A**) are representative of three independent experiments; data (**B**) are shown as mean ± SEM of three independent experiments.