nature immunology

Foxo1 links homing and survival of naive T cells by regulating L-selectin, CCR7 and interleukin 7 receptor

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Foxo transcription factors have a conserved role in the adaptation of cells and organisms to nutrient and growth factor availability. Here we show that Foxo1 has a crucial, nonredundant role in T cells. In naive T cells, Foxo1 controlled the expression of the adhesion molecule L-selectin, the chemokine receptor CCR7 and the transcription factor Klf2, and its deletion was sufficient to alter lymphocyte trafficking. Furthermore, *Foxo1* deficiency resulted in a severe defect in interleukin 7 receptor α-chain (IL-7Rα) expression associated with its ability to bind an *Il7r* enhancer. Finally, growth factor withdrawal induced a *Foxo1*-dependent increase in *Sell*, *Klf2* and *Il7r* expression. These data suggest that Foxo1 regulates the homeostasis and life span of naive T cells by sensing growth factor availability and regulating homing and survival signals.

Throughout adult life, the number and diversity of peripheral T cells depends on de novo cell development and cell division, balanced against programmed cell death. A growing number of studies show that this 'homeostasis' of T cells is controlled by cytokines, such as interleukin 7 (IL-7), as well as by interactions between T cell antigen receptor (TCR) and major histocompatibility complex (MHC)[1,2]. However, the cell-intrinsic factors responsible for the integration of environmental signals and the manner in which they manifest changes in cell populations remain poorly defined.

The Foxo subfamily of transcription factors has a highly conserved role in the regulation of life span, cell cycle progression, apoptosis, glucose metabolism and stress resistance by integrating information pertaining to the abundance of nutrients, growth factors and stress signals[3]. In mammals, the Foxo subfamily consists of four members: Foxo1 (A000944), Foxo3, Foxo4 and Foxo6. These factors often act as direct transcriptional activators or as coregulatory molecules through interactions with factors such as β-catenin, STAT3, Runx3, Smad3 or Smad4 (ref. 4). In response to growth factors such as insulin or cytokines, kinases downstream of phosphatidylinositol-3-OH kinase (PI(3)K), including Akt and/or SGK, phosphorylate Foxo proteins, resulting in their translocation to the cytoplasm and subsequent proteasomal degradation. Conversely, cell starvation and oxidative stress trigger the relocalization of Foxo members from the cytoplasm to the nucleus[5].

Foxo1 and Foxo3 have been detected in T and B cells[6]. After antigen or cytokine stimulation, they are rapidly phosphorylated and deactivated in a PI(3)K-dependent manner[7–11], whereas cytokine withdrawal causes their dephosphorylation and activation[8,12,13]. In T and B cell lines, overexpression of Foxo3 induces arrest in the G1 phase of the cell cycle and apoptosis, associated with induction of the cell cycle inhibitor $p27^{Kip1}$ and proapoptotic molecules FasL and Bim[8,10,13,14]. Moreover, Foxo1 and Foxo3 overexpression in the pro-B cell line Ba/F3 synergizes with the transcription factor EF1-δ to activate the transcription of *Ccng2* (encoding cyclin G2) and *Rbl2* (encoding retinoblastoma p130), two genes implicated in Foxo-dependent quiescence of fibroblasts[15,16].

Although these studies suggest that Foxo transcription factors promote apoptosis induced by quiescence or growth factor withdrawal in lymphocytes, the function of Foxo1 and Foxo3 in T cells remains poorly understood. Mice harboring a targeted deletion of *Foxo3* by retroviral insertion develop a mild lymphoproliferative syndrome associated with inflammatory lesions in multiple organs, $CD4^+$ T cell autoreactivity and increased cytokine production by T cells after *in vitro* restimulation[17]. However, phenotypic and functional analysis of T cells from two different strains of Foxo3-deficient mice did not reveal any spontaneous or autoimmune-driven T cell activation (A.S.D., D.R.B., Y.M.K., A. Babour, K. Arden *et al.*, unpublished observations; and refs. 18,19). In addition, studies involving acute deletion of *Foxo1*, *Foxo3* and *Foxo4* revealed a level of redundancy suggesting possible compensation between Foxo1 and Foxo3 in T cells[20,21]. Finally, the recent implications of Foxo1 and Foxo3 in the regulation of *Rag1* and *Rag2* expression and other aspects of B cell development revealed that these transcription factors could have unanticipated functions[22–24].

Here we report that, consistent with its preferential expression in lymphoid cells, conditional deletion of *Foxo1* substantially affected T cell homeostasis *in vivo*. Unexpectedly, whereas *Foxo1* deletion did not result in spontaneous T cell activation, Foxo1 was required to

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Received 9 July 2008; accepted 5 November 2008; published online 11 January 2009; doi:10.1038/ni.1689

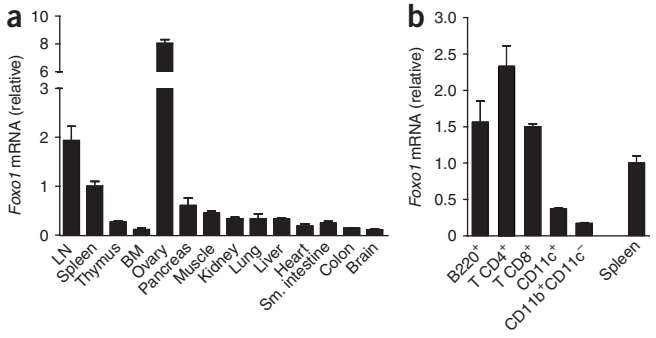

Figure 1 Foxo1 is preferentially expressed in lymphoid cells. (**a**) QPCR analysis of *Foxo1* mRNA expression in tissues from C57BL/6 mice. LN, lymph node; BM, bone marrow. (**b**) QPCR analysis of *Foxo1* mRNA expression in purified cell subsets from C57BL/6 mice lymph nodes and spleen. The abundance of *Foxo1* mRNA in each sample was normalized to that of *Hprt1* mRNA and then normalized to the amount obtained for the spleen (set to 1). Data in **a**,**b** are mean ± s.d. of duplicate samples. Results are representative of two independent experiments.

maintain naive T cell homeostasis through the regulation of several genes crucially involved in T cell trafficking and survival. Finally, we provide evidence that Foxo1 is key to negative feedback circuits that dynamically balance growth factor signaling with homing and survival of naive T cells.

RESULTS

Foxo1 is preferentially expressed in lymphoid cells

We compared the expression patterns of Foxo1 and Foxo3 in a variety of mouse tissues to explore their relative functional importance. *Foxo3* showed a ubiquitous expression pattern, whereas the highest expression of *Foxo1* mRNA was detected in the ovary, peripheral lymph nodes and spleen (**Fig. 1a** and data not shown). Quantitative PCR (QPCR) and immunoblot analyses of purified subsets revealed that *Foxo1* was highly expressed in $CD4^+$ T cells, $CD8^+$ T cells and B cells compared with dendritic cells ($CD11c^+$) and macrophages ($CD11b^+$ $CD11c^-$; **Fig. 1b**, **Supplementary Fig. 1a** online and data not shown). Consistent with these results, the expression of *Foxo1* and *Foxo3* mRNA reported in mouse and human expression databases[25] further showed that this pattern is conserved across species (**Supplementary Fig. 1b**). These observations support the idea of an essential role for Foxo1 in T and B cells.

Foxo1 deficiency impairs peripheral T cell homeostasis

Foxo1-null mice die at embryonic day 10.5 from defects in vasculogenesis[18,26]. To study the role of Foxo1 in T cell physiology *in vivo*, we crossed mice in which exon 2 of *Foxo1* is flanked by *lox*P sites (*Foxo1*$^{f/f}$ mice) to mice carrying the *Tg(Cd4-cre)1Cwi* transgene (*Cd4Cre* mice) to induce a T cell–specific recombination. Efficient and specific deletion of *Foxo1*, but not *Foxo3*, was evidenced by PCR of genomic DNA and immunoblot analysis, thereby allowing us to specifically study the effect of Foxo1 deficiency (**Supplementary Fig. 2** online).

Phenotypic analysis of various T cell subpopulations in 8- to 12-week-old *Foxo1*$^{f/f}$*-Cd4Cre* mice revealed a substantially higher proportion of activated-memory phenotype ($CD44^{hi}$) $CD4^+$ and $CD8^+$ T cells compared to littermate controls (**Fig. 2a**). Accordingly, we noted a considerable increase in the proportion of cytokine-secreting cells of the T helper type 1, 2 and 17 subsets and in $CD8^+$ T cells secreting both interferon-γ and tumor necrosis factor after restimulation *ex vivo* (**Supplementary Fig. 3a** online). Further analysis of the numbers of naive and activated-memory T cells indicated that this phenotype was the consequence of a reduced number of $CD44^{lo}CD4^+$ T cells, balanced by an expansion of the $CD44^{hi}$ population; for $CD8^+$ T cells, the deletion of *Foxo1* seemed to specifically affect the number of $CD44^{lo}$ cells (**Fig. 2b**). Finally, most Foxo1-deficient $CD4^+$ T cells showed characteristics typical of acutely activated T cells, as indicated by the concomitant increase in the proportion of cells expressing the early activation marker CD69 and the reduced expression of L-selectin (A001417) on $CD44^{hi}$ cells (**Supplementary Fig. 3b**). In contrast, no exaggerated increase in CD69-expressing cells was noted among $CD8^+$ T cells, and the majority of the $CD44^{hi}$ cells were L-selectin^{hi}, indicating that these cells were phenotypically related to central memory T cells (**Supplementary Fig. 3b**).

Consistent with a role in T cell quiescence, we reasoned that the increase in activated $CD4^+$ T cells in *Foxo1*$^{f/f}$*-Cd4Cre* mice could be the result of spontaneous activation. To analyze this, we generated

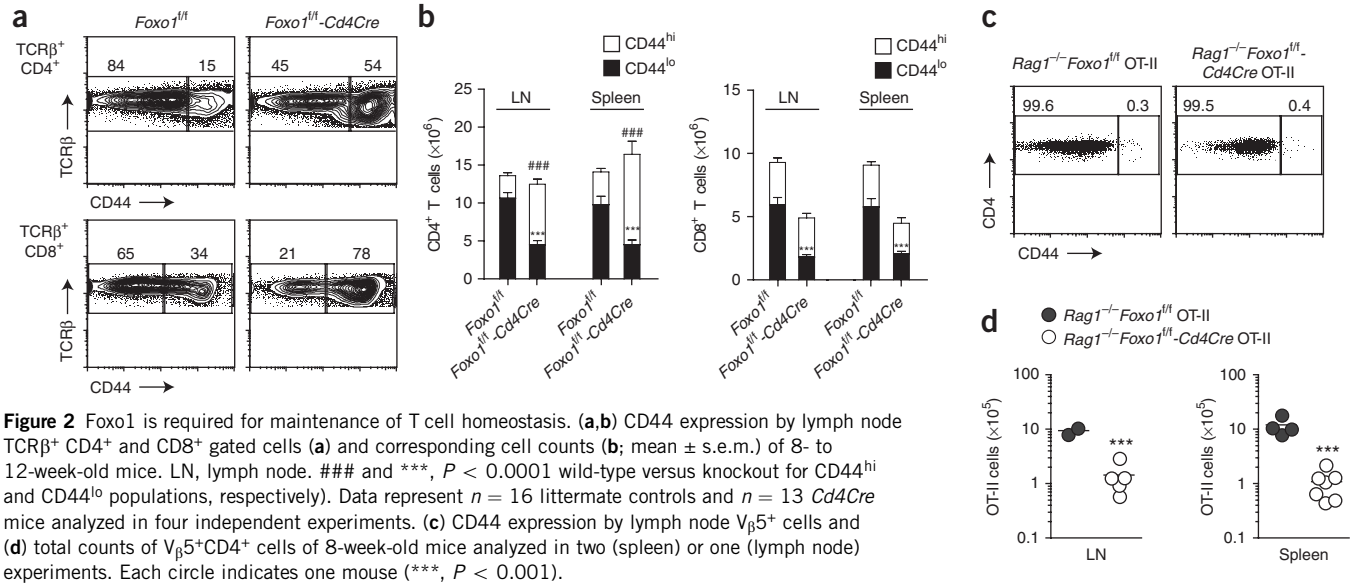

Figure 2 Foxo1 is required for maintenance of T cell homeostasis. (**a**,**b**) CD44 expression by lymph node $TCR\beta^+$ $CD4^+$ and $CD8^+$ gated cells (**a**) and corresponding cell counts (**b**; mean ± s.e.m.) of 8- to 12-week-old mice. LN, lymph node. ### and ***, $P < 0.0001$ wild-type versus knockout for $CD44^{hi}$ and $CD44^{lo}$ populations, respectively). Data represent $n = 16$ littermate controls and $n = 13$ *Cd4Cre* mice analyzed in four independent experiments. (**c**) CD44 expression by lymph node $V_\beta5^+$ cells and (**d**) total counts of $V_\beta5^+CD4^+$ cells of 8-week-old mice analyzed in two (spleen) or one (lymph node) experiments. Each circle indicates one mouse (***, $P < 0.001$).

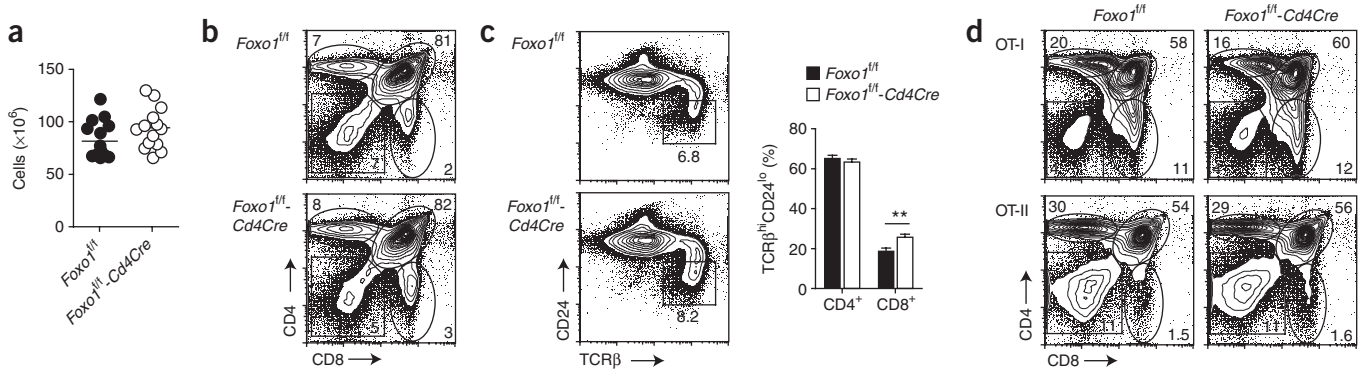

Figure 3 Foxo1 is dispensable for T cell development. (**a**) Total numbers of thymocytes. Each dot indicates one mouse. (**b**) Expression of CD4 and CD8 on thymocytes of the indicated genotypes. (**c**) Left, expression of TCRβ and CD24 on thymocytes of the indicated genotypes. Right, percentages of CD4$^+$ and CD8$^+$ single-positive cells within the population of mature TCRβ^{hi}CD24^{lo} thymocytes (mean ± s.e.m.) from 8-week-old mice. Data in **a**–**c** represent $n = 17$ littermate controls and $n = 14$ *Cd4Cre* mice, analyzed in five independent experiments (**, $P < 0.01$). (**d**) CD4 and CD8 expression on thymocytes from OT-II and OT-I transgenic mice. Data represent three to eight mice per genotype analyzed in two or three independent experiments.

Foxo1$^{f/f}$-*Cd4Cre* mice carrying the *Tg(TcraTcrb425)Cbn* transgene (called 'OT-II' here), which encodes a TCR specific for ovalbumin residues 323–339 in association with H-2A^b (ref. 27), and *Rag1*$^{-/-}$ alleles such that the resulting T cells did not recognize environmental or self-encoded antigens. V$_\beta$5$^+$CD4$^+$ cells from mice *Rag1*$^{-/-}$*Foxo1*$^{f/f}$-*Cd4Cre* OT-II showed a typical naive CD44^{lo}CD69$^-$ phenotype, with no spontaneous proliferation, as measured by incorporation of bromodeoxyuridine (**Fig. 2c** and data not shown). However, reminiscent of the lower number of naive CD44^{lo} T cells in *Foxo1*$^{f/f}$-*Cd4Cre* mice, *Foxo1* deletion in *Rag1*$^{-/-}$*Foxo1*$^{f/f}$-*Cd4Cre* OT-II mice resulted in a reduction of T cell numbers in secondary lymphoid organs to 10% of that in littermate controls (**Fig. 2d**). We concluded that Foxo1 has an essential and nonredundant role in T cell homeostasis independent of its potential role in quiescence. In addition, we hypothesized that the phenotype observed in *Foxo1*$^{f/f}$-*Cd4Cre* mice is caused in part by a specific reduction in the number of naive CD8$^+$ and CD4$^+$ T cells.

Foxo1 is dispensable for T cell development

Foxo1 has been implicated in B and T cell development[22–24,28]. We therefore examined whether the observed peripheral phenotype could arise from a defect in thymic differentiation. Analysis of the numbers and proportions of double-negative, double-positive and single-positive thymic subsets did not reveal any significant differences between *Foxo1*$^{f/f}$ and *Foxo1*$^{f/f}$-*Cd4Cre* mice (**Fig. 3a**,**b**), but we did note a trend in mice of the latter genotype toward a larger proportion of mature thymocytes (TCRβ^{hi}CD24^{lo}) and an increased frequency of CD8$^+$ cells (**Fig. 3c**). In addition, when Foxo1 deficiency was associated with transgenic expression of TCRs specific for MHC class I (*Tg(TcraTcrb)1100Mjb* transgene, or OT-I) or MHC class II (OT-II), there were again no significant differences in thymic cell subsets (**Fig. 3d**). Finally, we found that even early deletion of *Foxo1*, using the proximal *Lck* promoter to turn on Cre expression at the double-negative stage 3 of development (*Tg(Lck-cre)1Cwi*; called '*LckCre*' here), had no effect on the principal thymic cell populations. The phenotype of these mice was similar to that of the *Foxo1*$^{f/f}$-*Cd4Cre* mice, with a trend toward a greater number of mature CD8$^+$ T cells compared with *Foxo1*$^{+/+}$-*Lck Cre* mice (**Supplementary Fig. 4** online). Consistent with the low expression of Foxo1 in the thymus (**Fig. 1a**) and reports showing that Foxo1 is expressed in only the most mature thymocytes (ref. 29 and http://www.immgen.org/index_content.html), these results indicate that the reduced number of peripheral, naive T cells in *Foxo1*$^{f/f}$-*Cd4Cre* mice does not stem from a lack of progression through thymic development.

Foxo1 controls naive T cell homing

Initial phenotypic analysis of Foxo1-deficient T cells revealed a consistent reduction of L-selectin expression on naive CD4$^+$ and CD8$^+$ CD44^{lo} T cells compared with wild-type cells, whereas CD11a (LFA1α, α$_L$ integrin), another receptor involved in lymph node migration, was unaffected (**Fig. 4a** and data not shown). In addition, although CD44 expression was unaffected, mature (TCRβ^{hi}) single-positive thymocytes from *Foxo1*$^{f/f}$-*Cd4Cre* mice showed reduced surface expression of L-selectin, as did T cells from *Rag1*$^{-/-}$*Foxo1*$^{f/f}$-*Cd4Cre* OT-II mice (**Fig. 4b** and **Supplementary Fig. 5** online). These results suggested that Foxo1 deficiency could alter naive T cell homing.

To further analyze this effect, we used mice expressing a chimeric *Esr1-cre* gene recombined into the ubiquitously expressed *Rosa26* locus[30] (*Gt(ROSA)26Sor*; called '*ERCre*' here). After treatment with tamoxifen, the estrogen receptor (ER)-Cre fusion protein, normally sequestrated in the cytoplasm, translocates to the nucleus, allowing Cre-mediated deletion of *lox*P-flanked alleles. We treated *Foxo1*$^{f/f}$ and *Foxo1*$^{f/f}$-*ERCre* mice for 5 d with tamoxifen and then rested them for 5 d. QPCR and immunoblot analysis of purified T cells showed that acute activation of the ER-Cre fusion protein resulted in efficient reduction of Foxo1 mRNA and protein, whereas Foxo3 mRNA expression remained unaltered (**Supplementary Fig. 6a**,**b** online). Notably, this short-term deficiency did not significantly affect the proportion of naive and activated-memory T cell populations (**Supplementary Fig. 6c**), indicating that the phenotype observed in adult *Foxo1*$^{f/f}$-*Cd4Cre* mice requires prolonged insufficiency of Foxo1. In addition, this experimental system allowed us to exclude the contribution of potential secondary effects induced by excessive T cell activation, a lymphopenic environment or abnormal thymic T cell maturation. Similar to the effect of *Cd4Cre*-mediated *Foxo1* deletion, acute tamoxifen-mediated deletion of *Foxo1* induced a 60% reduction of L-selectin protein expression on CD44^{lo} CD4$^+$ and CD8$^+$ T cells, associated with a 50% reduction of *Sell* mRNA (L-selectin) in purified lymph node T cells (**Fig. 4c**,**d**).

Further analysis of homing receptor expression on CD44^{lo} T cells in *Foxo1*$^{f/f}$-*ERCre* and *Foxo1*$^{f/f}$-*Cd4Cre* mice revealed that *Foxo1* deficiency also affects both protein and mRNA expression of CCR7 (A000630; **Fig. 4c**,**d**, **Supplementary Fig. 5** and data not shown). Because both L-selectin and CCR7 expression are dependent on the

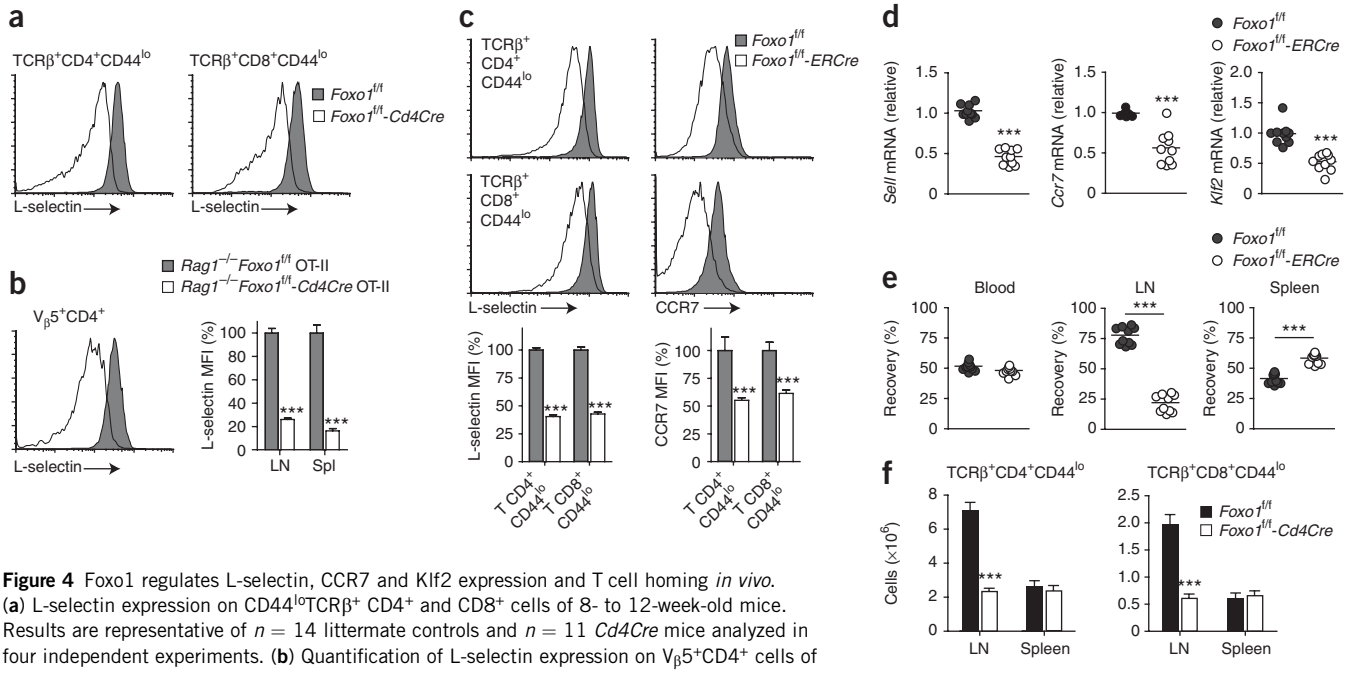

Figure 4 Foxo1 regulates L-selectin, CCR7 and Klf2 expression and T cell homing *in vivo*. (**a**) L-selectin expression on $CD44^{lo}TCR\beta^+$ $CD4^+$ and $CD8^+$ cells of 8- to 12-week-old mice. Results are representative of $n = 14$ littermate controls and $n = 11$ *Cd4Cre* mice analyzed in four independent experiments. (**b**) Quantification of L-selectin expression on $V_\beta5^+CD4^+$ cells of 8-week-old mice (mean ± s.e.m.). LN, lymph node. Data represent $n = 4$ littermate controls and $n = 7$ *Cd4Cre* mice analyzed in two independent experiments (***, $P < 0.0001$). MFI, mean fluorescence intensity. (**c–e**) *Foxo1*$^{f/f}$*-ERCre* mice and littermate controls ($CD45.2^+$) were treated for 5 d with tamoxifen and rested for 5 d. (**c**) Quantification of L-selectin and CCR7 expression on lymph node $CD44^{lo}TCR\beta^+$ $CD4^+$ and $CD8^+$ cells (mean ± s.e.m.). Data represent $n \geq 4$ mice per genotype, analyzed in two to three independent experiments (***, $P < 0.0001$). (**d**) QPCR analysis of *Sell*, *Ccr7* and *Klf2* mRNA expression, normalized to *Hprt1* mRNA, in purified lymph node T cells. Each circle indicates one mouse (***, $P < 0.0001$). (**e**) lymph node T cells were purified, and one of two populations was labeled with CFSE. The two populations were mixed at a 1:1 ratio and injected into C57BL/6 $CD45.1^+$ mice (10×10^6 cells per mouse). Donor cell recovery was analyzed 18 h later in peripheral blood, lymph nodes and spleen ($CD45.2^+$-gated $CFSE^+$ versus $CFSE^-$ cells). Each circle indicates one host mouse. Results are from two independent experiments (***, $P < 0.0001$). (**f**) Number of naive T cells (mean ± s.e.m.) in 3-week-old mice. Data represent $n = 6$ littermate controls and $n = 9$ *Cd4Cre* mice analyzed in two independent experiments (***, $P < 0.0001$).

transcription factor Klf2 (ref. 31), we examined *Klf2* mRNA expression. The results showed that tamoxifen treatment caused a 50% decrease in *Klf2* expression in T cells from *Foxo1*$^{f/f}$*-ERCre* mice relative to that of T cells from *Foxo1*$^{f/f}$ mice (**Fig. 4d**).

Finally, we investigated the functional effects of *Foxo1* deletion on T cell trafficking *in vivo*. We purified lymph node T cells from tamoxifen-treated *Foxo1*$^{f/f}$ and *Foxo1*$^{f/f}$*-ERCre* mice, labeled them with the cytosolic dye CFSE, mixed them at a ratio of 1:1 and transferred them into wild-type CD45.1 recipients. The number of Foxo1-deficient T cells in lymph nodes was severely impaired. After 18 h, although we recovered an equal proportion of transferred T cells from blood, we found that the ability of Foxo1-deficient T cells to migrate into the lymph nodes was considerably impaired relative to that of Foxo1-sufficient cells (**Fig. 4e**). Consistent with the altered migratory properties of L-selectin– and CCR7-deficient T cells, as well as pertussis toxin–treated T cells[32–34], Foxo1-deficient T cells accumulated in the spleen (**Fig. 4e**). Together, these data show that Foxo1 regulates the expression of L-selectin, CCR7 and Klf2 and controls homing of naive T cells *in vivo*.

Foxo1 is required for naive T cell survival

L-selectin and CCR7 deficiencies are associated with decreased T cell numbers in the lymph nodes and normal or increased T cell numbers in the blood and spleen[32,33]; in contrast, Klf2 deficiency induces a general decrease in the number of peripheral T cells owing to retention of mature T cells in the thymus and abnormal homing[31,35]. Consistent with an important role for Foxo1 in naive T cell homing and with the acute deletion experiments, 3-week-old *Foxo1*$^{f/f}$*-Cd4Cre* mice showed a specific reduction in the number of naive lymph node T cells (**Fig. 4f**). Our results also showed that *Foxo1* deletion does not significantly affect thymic cell numbers, but leads to a similarly lower number of naive $CD44^{lo}$ T cells in the lymph nodes and spleen of adult *Foxo1*$^{f/f}$*-Cd4Cre* mice, *Foxo1*$^{f/f}$*-LckCre* mice and *Foxo1*$^{f/f}$*-ERCre* mice 5 weeks after tamoxifen treatment (**Figs. 2** and **3** and data not shown). Moreover, compared with *Foxo1*$^{f/f}$ mice, adult *Foxo1*$^{f/f}$*-Cd4Cre* mice showed a substantial decrease in the proportion and relative number of circulating blood T cells, relative to wild-type, and those present were mostly $CD44^{hi}$ (**Fig. 5a** and data not shown). We therefore considered whether, in addition to the role of Foxo1 in the regulation of T cell trafficking, prolonged loss of Foxo1 might also affect T cell survival.

To test this hypothesis, we adoptively transferred wild-type T cells with *Foxo1*$^{f/f}$ or *Foxo1*$^{f/f}$*-Cd4Cre* T cells from adult mice into wild-type hosts. As opposed to naive Foxo1-sufficient T cells, which were normally maintained, the proportion of naive $CD44^{lo}$ $CD4^+$ and $CD8^+$ T cells from *Foxo1*$^{f/f}$*-Cd4Cre* mice rapidly decreased in all organs tested (**Fig. 5b** and data not shown). Notably, we consistently recorded a diminished T cell recovery in lymph nodes compared to spleen and blood, suggestive of altered T cell homing. The impaired maintenance of Foxo1-deficient cells was not caused by Cre-induced toxicity or rejection, as *Foxo1*$^{f/+}$*-Cd4Cre* T cells were normally maintained after transfer (data not shown). Additionally, generation of mixed bone marrow chimeras showed that *Foxo1*$^{f/f}$*-Cd4Cre* bone marrow cells were unable to reconstitute a normal T cell compartment (**Fig. 5c**).

T cell survival is dependent on balanced expression of the proapoptotic protein Bim and prosurvival proteins including Bcl-2 and

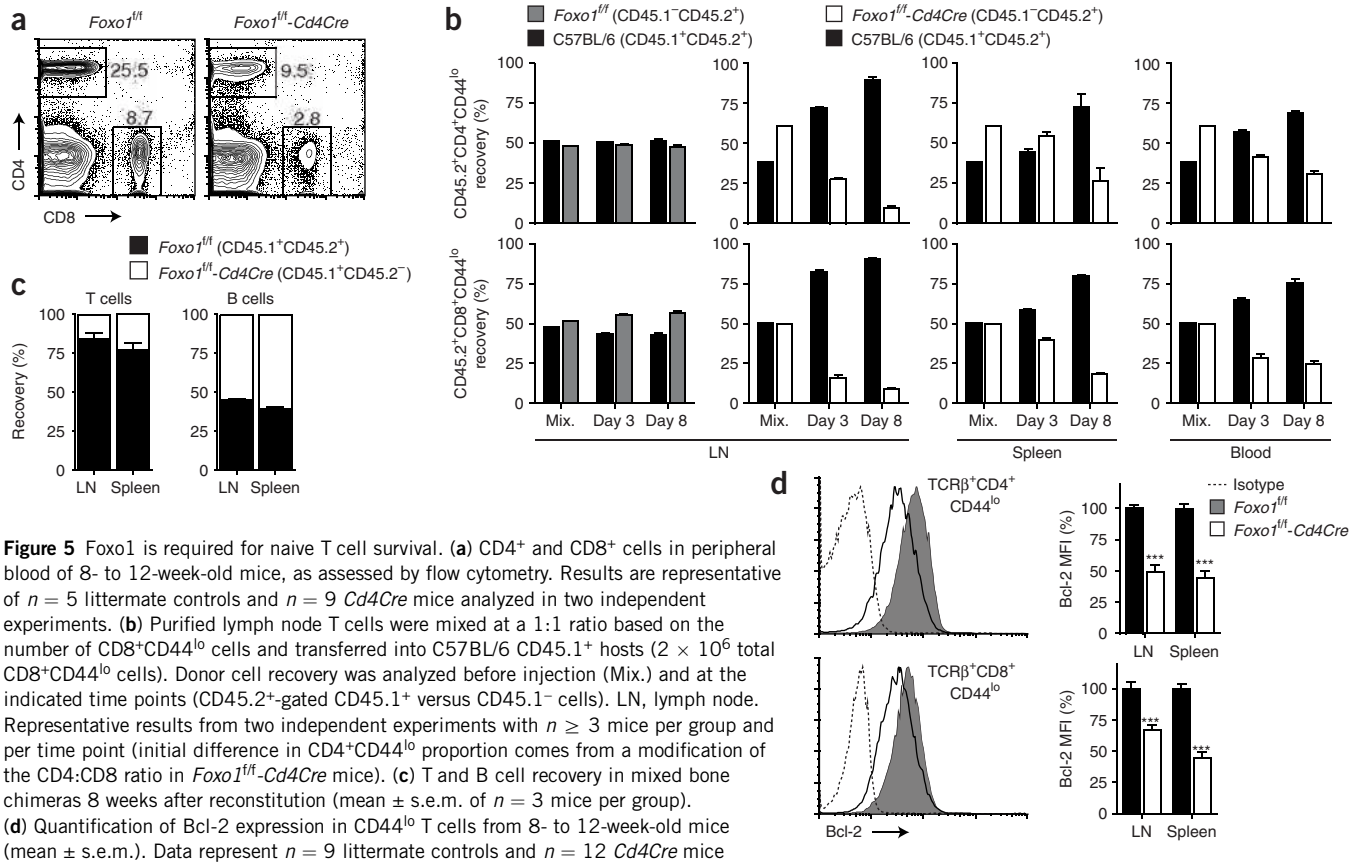

Figure 5 Foxo1 is required for naive T cell survival. (**a**) CD4$^+$ and CD8$^+$ cells in peripheral blood of 8- to 12-week-old mice, as assessed by flow cytometry. Results are representative of $n = 5$ littermate controls and $n = 9$ *Cd4Cre* mice analyzed in two independent experiments. (**b**) Purified lymph node T cells were mixed at a 1:1 ratio based on the number of CD8$^+$CD44^{lo} cells and transferred into C57BL/6 CD45.1$^+$ hosts (2×10^6 total CD8$^+$CD44^{lo} cells). Donor cell recovery was analyzed before injection (Mix.) and at the indicated time points (CD45.2$^+$-gated CD45.1$^+$ versus CD45.1$^-$ cells). LN, lymph node. Representative results from two independent experiments with $n \geq 3$ mice per group and per time point (initial difference in CD4$^+$CD44^{lo} proportion comes from a modification of the CD4:CD8 ratio in *Foxo1*$^{f/f}$*-Cd4Cre* mice). (**c**) T and B cell recovery in mixed bone chimeras 8 weeks after reconstitution (mean ± s.e.m. of $n = 3$ mice per group). (**d**) Quantification of Bcl-2 expression in CD44^{lo} T cells from 8- to 12-week-old mice (mean ± s.e.m.). Data represent $n = 9$ littermate controls and $n = 12$ *Cd4Cre* mice analyzed in two independent experiments (***, $P < 0.0001$).

Bcl-x_L[1,36]. Although previous studies have shown a role for Foxo3 as a transcriptional regulator of Bim expression in T cells, we did not detect changes in Bim mRNA expression after acute deletion of *Foxo1* (**Supplementary Fig. 6d**). However, we did find decreased expression of Bcl-2 in CD44^{lo} CD4$^+$ and CD8$^+$ T cells (**Fig. 5d**). These results collectively indicate that Foxo1 is required to maintain the expression of Bcl-2 and the survival of naive T cells *in vivo*.

Foxo1 controls IL-7Rα expression in naive T cells

IL-7 is required for the survival of naive T cells *in vivo*, and one consequence of IL-7R (A001267) signaling is Bcl-2 induction[1,2,37,38]. As Foxo transcription factors are inactivated after cytokine stimulation and thus are unlikely to be directly responsible for this effect, we considered that *Foxo1* deletion could alter the expression of IL-7R. Wild-type, naive CD44^{lo} CD4$^+$ and CD8$^+$ T cells expressed high amounts of both the IL-7Rα chain (CD127) and the common cytokine receptor γ-chain (CD132) constituting the IL-7R. Notably, IL-7Rα expression was severely impaired on CD44^{lo} CD4$^+$ and CD8$^+$ T cells from *Foxo1*$^{f/f}$*-Cd4Cre* mice, whereas expression of γ-chain was unaffected (**Fig. 6a**). Consistent with this phenotype, addition of IL-7 did not rescue naive Foxo1-deficient T cells from death induced by *ex vivo* growth factor withdrawal, and STAT5 phosphorylation induced by IL-7 was markedly reduced (**Fig. 6b** and data not shown).

The expression of IL-7Rα was also impaired on thymic, mature T cells from *Foxo1*$^{f/f}$*-Cd4Cre* mice (**Supplementary Fig. 5**). We thus wished to determine whether the impaired expression of IL-7Rα on peripheral, naive T cells arose from a blockade of its expression during T cell maturation or whether Foxo1 was required for continuous expression. We treated *Foxo1*$^{f/f}$ and *Foxo1*$^{f/f}$*-ERCre* mice with tamoxifen and followed the expression of IL-7Rα on peripheral CD44^{lo} T cells over time. Acute deletion of *Foxo1* induced a rapid and profound downregulation of IL-7Rα expression associated with a significant reduction of *Il7r* mRNA, but not *Il2rg* mRNA, in purified lymph node T cells (**Fig. 6c**,**d**).

Among the genes we found to be affected by *Foxo1* deletion, *Il7r* showed the most pronounced regulation. We therefore sought to determine whether Foxo1 directly regulates *Il7r* expression. Genomic alignment of the *Il7r* locus across several mammalian species indicated the presence of three evolutionarily conserved noncoding regions (ECRs) upstream of the transcription initiation site, including one corresponding to the defined promoter region (**Supplementary Fig. 7** online and ref. 39). Detailed bioinformatic analysis of transcription factor binding sites in each of these regions revealed the presence of a highly conserved Foxo binding sequence in ECR2 located 3.5 kb upstream of the transcription initiation site (**Fig. 6e** and **Supplementary Fig. 7**). To determine whether Foxo1 can directly bind within the *Il7r* locus, we conducted chromatin immunoprecipitation experiments using primer sets designed to amplify regions located in each of these ECRs. In purified lymph node T cells, Foxo1 bound to the *Il7r* locus specifically within the region containing this putative binding site (**Fig. 6f**). Collectively, these data strongly suggest that Foxo1 regulates IL-7Rα expression by binding directly to this ECR in the *Il7r* locus.

Dynamic regulation of Foxo1 activity

Growth factor withdrawal leads to Foxo dephosphorylation and increased activity, whereas TCR or cytokine (including IL-7)

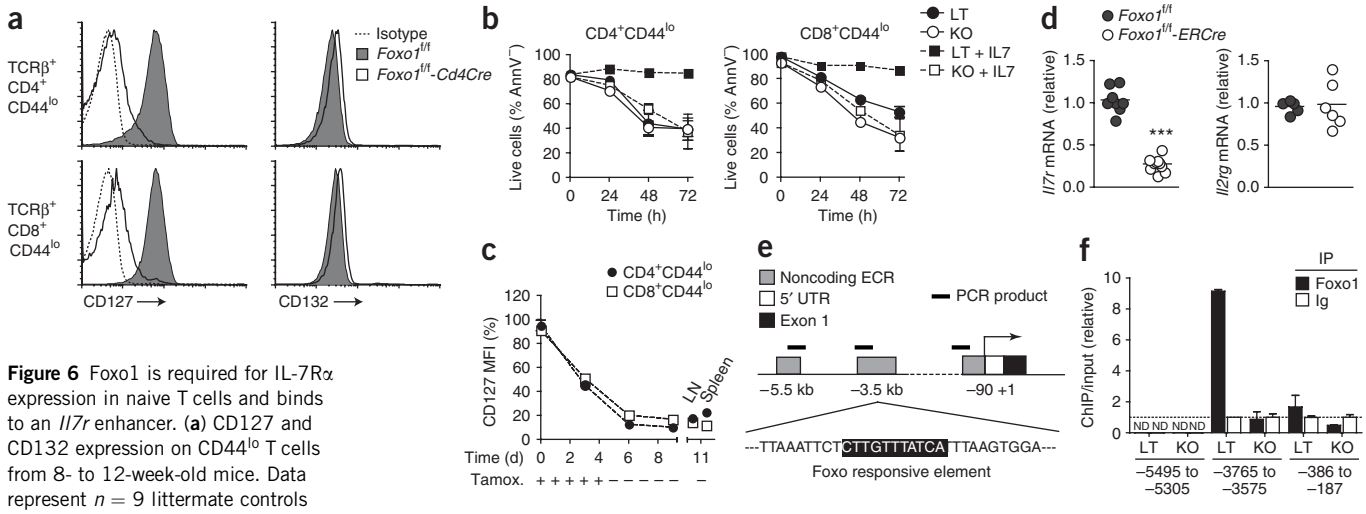

Figure 6 Foxo1 is required for IL-7Rα expression in naive T cells and binds to an *Il7r* enhancer. (**a**) CD127 and CD132 expression on CD44^{lo} T cells from 8- to 12-week-old mice. Data represent $n = 9$ littermate controls (LT) and $n = 10$ *Cd4Cre* mice (KO) analyzed in three independent experiments. (**b**) Lymph node (LN) cells were cultured in medium supplemented or not with IL-7 for 3 d, and the proportion of live (annexin V–negative, AnnV$^-$) CD44^{lo} CD4$^+$ and CD8$^+$ T cells was measured by flow cytometry at the indicated time points (mean ± s.d. of triplicate cultures). Results are representative of three independent experiments. (**c**) Quantification of CD127 expression on CD44^{lo} CD4$^+$ and CD8$^+$ T cells after tamoxifen treatment in *Foxo1*$^{f/f}$*-ERCre* mice (mean ± s.e.m.). MFI, mean fluorescence intensity. Results are representative of three independent experiments with $n = 7$–11 mice per time point and per genotype. (**d**) QPCR analysis of *Il7r* and *Il2rg* mRNA expression in purified lymph node T cells on day 11 after the beginning of tamoxifen treatment. Each dot indicates one mouse (***, $P < 0.0001$). (**e**) *Il7r* locus. (**f**) Chromatin immunoprecipitation analysis of Foxo1 binding to the *Il7r* locus in purified lymph node T cells from littermate controls and *Cd4Cre* mice. Results are relative to the value obtained for the control immunoprecipitation (Ig), with Foxo1-sufficient T cells set as 1 (mean ± s.d. of duplicate samples). ND, not detected. Results are representative of four independent experiments.

stimulation induces Foxo phosphorylation and decreased transcription of target genes. This finding is consistent with the observation that T cells cultured in the absence of growth factors show increased IL-7Rα expression, whereas stimulation with IL-7, IL-2, IL-4, IL-6 or IL-15 decreases IL-7Rα expression[40,41]. Accordingly, overnight culture of *Foxo1*-sufficient T cells in culture medium without added growth factors resulted in a strong increase in both IL-7Rα surface expression and *Il7r* mRNA, an effect that was completely inhibited by the addition of IL-7 (**Fig. 7a**,**b** and data not shown). A deficiency in Foxo1 completely prevented this increase, and the addition of IL-7 had no effect (**Fig. 7a**,**b**). Moreover, overnight growth factor starvation of lymph node T cells was associated with recruitment of Foxo1 to the *Il7r* locus, an effect inhibited by the addition of IL-7 (**Fig. 7c**).

Previous studies have shown that culturing T cells in absence of any stimulation also induces an increase in the surface expression of L-selectin on both CD4$^+$ and CD8$^+$ T cells[42], although the expression of CCR7 does not seem to be affected[43]. Considering our previous results, we analyzed the role of Foxo1 in these effects and observed that growth factor withdrawal induces a similar Foxo1-dependent increase in the expression of *Sell* and *Klf2* mRNA in naive T cells (**Fig. 7d**). This increase was again prevented by the addition of IL-7. Notably, *Ccr7* mRNA expression was unaffected by any of these culture conditions, independent of Foxo1 expression. These results also revealed that the expression of L-selectin, CCR7 and Klf2 does not require IL-7R signaling, thus showing that the defective expression of these molecules in Foxo1-deficient T cells is not secondary to the defective IL-7Rα expression. Furthermore, L-selectin and CCR7 were not decreased on CD44^{lo} T cells from *Il7r*$^{-/-}$ mice (**Supplementary Fig. 8** online), in agreement with the phenotype of TCR-transgenic *Il2rg*$^{-/-}$ T cells[44].

As the expression of *Il7r* and *Sell* increased in wild-type T cells but not Foxo1-deficient T cells *ex vivo*, this suggested that basal inhibition of Foxo1 *in vivo* limits their expression. Indeed, acute deletion of the PI(3)K-dependent pathway inhibitor PTEN was sufficient to decrease the expression of IL-7Rα and L-selectin in naive T cells *in vivo* (**Supplementary Fig. 9a**,**b** online). Moreover, *Pten* deletion prevented IL-7Rα upregulation after cytokine withdrawal *ex vivo* and enhanced its downregulation after IL-7 stimulation (**Supplementary Fig. 9c**),

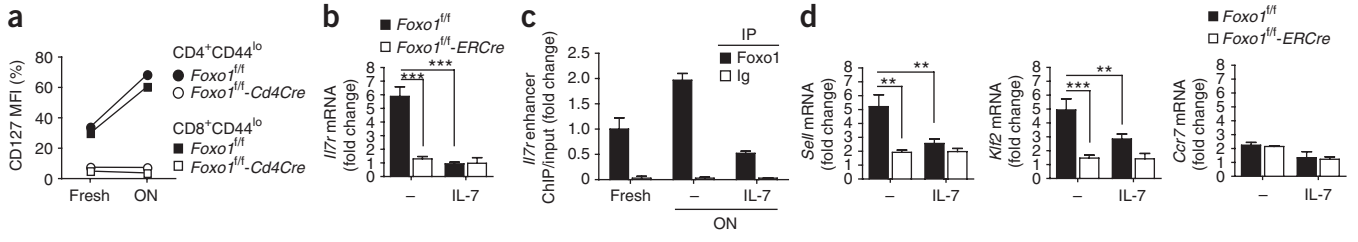

Figure 7 Foxo1-mediated control of Il-7Rα and trafficking receptors after cell starvation. (**a**) CD127 expression on lymph node T cells freshly isolated and rested overnight (ON) in medium (mean ± s.d. of triplicate cultures). (**b–d**) *Foxo1*$^{f/f}$*-ERCre* mice and littermate controls were treated with tamoxifen for 5 d and rested for 3 d. Lymph node T cells were then purified and cultured overnight in medium supplemented with IL-7 (10 ng/ml) as indicated. (**b**) QPCR analysis of *Il7r* mRNA, normalized to *Hprt* mRNA, after overnight culture. **c**, Chromatin immunoprecipitation analysis of Foxo1 binding to *IL7r* ECR2. (**d**) QPCR analysis of *Sell*, *Klf2* and *Ccr7* mRNA. Results are presented as fold change (mean ± s.d. of triplicate cultures) relative to the value obtained for freshly isolated T cells set to 1. Results are representative of two (**a**,**c**) or three (**b**,**d**) independent experiments (**, $P < 0.01$; ***, $P < 0.0001$).

thus showing that unrestrained activation of the PI(3)K pathway inhibits IL-7Rα expression. Collectively, these results indicate that pathways controlling homing and survival of naive T cells are coordinately and dynamically regulated by growth factor availability through the transcription factor Foxo1.

DISCUSSION

Maintenance of lymphocyte homeostasis is crucial to prevent immunopathology while promoting the generation of protective immunity. Studies over the past 30 years have shown that the T cell population is regulated by homeostasis, albeit with a high degree of plasticity[45,46]. More recent studies have established the importance of TCR-MHC interactions in maintaining T cell viability and of IL-7 as the essential T cell survival cytokine[47–50]. However, the cell-intrinsic factors acting as the 'control center' to integrate these environmental signals and determine the appropriate response remain elusive. To achieve this function, T cells may use mechanisms universally used in eukaryotes. In particular, the Foxo family of transcription factors has a highly conserved role in the regulation of cellular and organismal metabolism depending on nutrient or growth factor availability.

To date, most studies on Foxo transcription factors have shown their involvement in quiescence of naive T cells and apoptosis induced by growth factor withdrawal[7,9,51,52], but these hypotheses have not been exhaustively tested *in vivo*. Our experiments show that *Foxo1* deletion alone does not result in spontaneous T cell proliferation, increased numbers of cells, modification of Bim expression or increased resistance to apoptosis induced by growth factor withdrawal. Rather, we provide compelling evidence that Foxo1 plays a crucial role in naive T cell homeostasis by promoting homing to lymph nodes and survival of naive T cells. However, as Foxo1-deficient T cells still express Foxo3, these results do not rule out the likely possibility of redundancy in the regulation of target genes, which may include those involved in quiescence and apoptosis.

Deletion of *Foxo1* resulted in a specific reduction of naive T cells in the lymph nodes of young *Foxo1*$^{f/f}$-*Cd4Cre* mice, associated with decreased cell surface expression of L-selectin and CCR7 and diminished expression of *Sell*, *Ccr7* and *Klf2* mRNAs. Consistent with these data, naive Foxo1-deficient T cells showed defective homing after transfer *in vivo*. Naive T cells were not completely absent from peripheral lymph nodes, and thymus egress seemed fairly unperturbed. The expression of L-selectin, CCR7 and Klf2 was diminished in Foxo1-deficient naive T cells but not entirely abrogated, indicating that other mechanisms are involved in the regulation of these genes. Furthermore, these results suggest that the remaining expression may be sufficient to allow thymic egress and homing of naive T cells into lymph nodes, albeit at a lower rate. Notably, a previous study showed that the inhibition of Klf2, L-selectin and CCR7 expression is controlled by PI(3)K signaling during T cell activation[53]. We thus consider the possibility of a redundancy between Foxo1 and Foxo3. In support of this hypothesis, preliminary results indicate that loss of both Foxo1 and Foxo3 affects T cell egress from the thymus (Y.M.K. and S.M.H., unpublished observations). However, the transcriptional regulation of Klf2, L-selectin and CCR7 is, at least partially, rapamycin sensitive, suggesting the existence of alternative PI(3)K-dependent regulatory mechanisms that coexist with Foxo1 to control expression[53].

Klf2 deficiency is associated with defective L-selectin expression, and Klf2 can transactivate the *Sell* promoter in reporter assays *in vitro*[31,54]. The decreased L-selectin expression in Foxo1-deficient T cells can therefore originate from the Foxo1-mediated control of *Klf2* transcription. Indeed, one study reports that overexpression of a constitutively active form of human FOXO1 induces the expression of L-selectin and KLF2, and FOXO1 binds to the *KLF2* promoter in human T cells[55]. Consistent with our results, this study also showed that overexpression of active FOXO1 in a Jurkat T cell line leads to the induction of *CCR7* mRNA expression. As *KlF2* deficiency affects the cell surface expression, but not the transcription, of *Ccr7* (refs. 31,35), these data indicate that Foxo1 may also act through other regulatory mechanisms to regulate CCR7 expression and thus naive T cell homing. Supporting this view and further indicating the uncoupled regulation of L-selectin and CCR7, previous work and our results show that T cell starvation induces a parallel Foxo1-dependent increase of both *Sell* and *Klf2* transcription, whereas *Ccr7* expression is unchanged.

The results presented here show that Foxo1 further links the regulation of homing with the regulation of cell viability by controlling IL-7Rα expression in naive T cells. *Foxo1*$^{f/f}$-*Cd4Cre* mice represent the first model with such a profound defect in IL-7Rα expression on peripheral naive T cells, without the complications associated with T cell development in mice deficient in IL-7 or IL-7Rα. Despite the well-known role of IL-7 signaling in naive T cell survival, we detected a substantial number of naive T cells in secondary lymphoid organs of *Foxo1*$^{f/f}$-*Cd4Cre* mice. Consistent with our results, anti-IL7Rα treatment *in vivo* induced a comparable decrease in T cell numbers[29]. In addition, as observed in *Il7r*$^{-/-}$ mice[49], the expression of Bcl-2 is not completely abrogated. One conclusion is that other pathways contribute to Bcl-2 expression and naive T cell survival *in vivo*. Of note, we observed that expression of the OT-II TCR on Foxo1-deficient T cells accentuates the decrease in naive T cell numbers, consistent with a role for TCR-MHC interactions.

Despite the loss of IL-7Rα expression and paucity of naive T cells, we observed normal or higher numbers of activated-memory phenotype cells in *Foxo1*$^{f/f}$-*Cd4cre* mice. These results therefore indicate that other growth factors, such as IL-15, could substantially contribute to the survival of these cells; however, the TCR-dependent expansion and increased proportion of CD4$^+$ T cells expressing the early-activation marker CD69 suggest that Foxo1 has other important functions in the regulation of T cell homeostasis.

The expression of IL-7Rα is regulated by growth factor–induced negative feedback[41]. Growth factor withdrawal resulted in Foxo1 recruitment to a region of the *Il7r* locus previously characterized for glucocorticoid receptor–dependent enhancer activity[56] and was associated with Foxo1-dependent IL-7Rα expression. At least for CD8$^+$ T cells, this regulation may be further modulated by another factor, GFI[41], but these data establish an essential role for Foxo1 in the regulation of IL-7Rα by negative feedback.

One theory regarding the logic underlying the role of Foxo1 in the common control of homing receptors and IL-7Rα is that the amount of available IL-7 is fixed and limiting for T cell survival; and this IL-7 concentration determines, in part, the size of the T cell population[37]. For example, at least some IL-7 transgenic mice have greatly expanded numbers of T cells[57,58]. Also, naive T cells compete for limited self-peptide–MHC complexes to maintain their survival[47,59], and both IL-7Rα and TCR signaling activate PI(3)K and Akt, resulting in the inactivation of Foxo factors[9,60,61]. Furthermore, as we show here, such signaling inhibits IL-7Rα, L-selectin and CCR7 expression, so those T cells receiving the most stimulation will subsequently be disadvantaged in two ways. First, they will home to secondary lymphoid organs at a reduced rate—and this in itself is a requirement for survival[43,62]; second, they will compete less effectively for limited IL-7. This negative feedback is predicted to cause an oscillation of survival and homing signals and prevent the most avaricious T cells from dominating the population. Thus, we predict that this feedback circuit is crucial to

prevent narrowing of the naive repertoire in the periphery once generated and selected in the thymus. Together, the results presented here reveal an unanticipated connection between homing and survival of naive T cells through the transcription factor Foxo1 and emphasize its importance in the regulation of naive T cell homeostasis.

METHODS

Mice and tamoxifen treatment. C57BL/6, C57BL/6 CD45.1$^+$, *Cd4Cre*, *LckCre*, *Rag1*$^{-/-}$, OT-II and OT-I mice were maintained in pathogen-free conditions. *ERCre* mice were provided by T. Ludwig[30], and *Pten*$^{f/f}$*-ERCre* mice were provided by C. Murre. Gene-trapped Foxo3-deficient (*Foxo3*^{Kca}) mice were provided by K. Arden and backcrossed ten times to C57BL/6 mice. *Foxo1*$^{f/f}$ mice have been described[20,21]. *H-2*^{b} homozygous F_2 mice from *Foxo1*$^{f/f}$ (FVB/N, H-2^q) mice crossed to *Cd4Cre* (C57BL/6, H-2^b) or *ERCre* (C57BL/6, H-2^b) mice were used to set up breeding pairs. Analysis of *Foxo1*$^{f/+}$*-Cd4Cre* mice did not reveal any significant phenotypic differences from *Foxo1*$^{f/f}$ mice, ruling out a Cre-mediated effect or hemizygous gene dosage effect as a cause of the *Foxo1*$^{f/f}$*-Cd4Cre* mice phenotype (data not shown). Mice used in short-term T cell transfer experiments were generated from *Foxo1*$^{f/+}$ mice backcrossed six times to C57BL/6 mice. CD45.1$^+$CD45.2$^+$ C57BL/6 mice were produced by breeding C57BL/6 mice with CD45.1$^+$ C57BL/6 mice. All procedures were approved by the Animal Care and Use Committee of the University of California, San Diego. *ERCre*-mediated deletion of floxed alleles was induced by intraperitoneal injection of 1 mg of tamoxifen (Sigma) emulsified in 200 µl of sunflower seed oil (Sigma) every day for 5 d.

Flow cytometry. Cell suspensions prepared from the indicated organs were incubated for 20 min at 4 °C in PBS containing 1% FCS, 2 mM EDTA, 0.01% NaN_3 and the indicated fluorochrome-conjugated antibodies, in the presence of an optimal concentration of 2.4G2 hybridoma culture supernatant (antibody to mouse FcγRII/III). CCR7 staining was done at 37 °C for 30 min with phycoerythrin-conjugated antibody to mouse CCR7 (eBioscience). All intracellular staining was done with BD Cytofix/Cytoperm (BD Biosciences). Antibodies were purchased from eBioscience or BD PharMingen; clone identifiers are listed in **Supplementary Table 1** online. Data were collected on a FACSCalibur (BD Biosciences) and analyzed with FlowJo software (Tree Star). Mean fluorescence intensity quantifications across experiments were assessed by normalizing mean fluorescence intensity values obtained for each mouse, with the mean of the values obtained for control mice set as 100% for each experiment or time point.

Immunoblot. Whole-cell extracts were resolved on 4–12% SDS-PAGE gels (Invitrogen) and transferred to a polyvinylidene fluoride membrane (Millipore) using a semidry transfer cell (Bio-Rad). Blots were blocked and incubated with the primary antibody at 4 °C overnight, followed by a 2-h incubation at 25 °C with the appropriate horseradish peroxidase–conjugated secondary antibody. Primary antibodies to the following molecules were used: Foxo1 (rabbit polyclonal; 9462; Cell Signaling Technology), phospholipase C-γ (Upstate Biotechnology) and β-tubulin (Upstate Biotechnology). Rabbit polyclonal antibody to Foxo3 was provided by A. Brunet. The specificity of this antibody was confirmed by the inclusion of cells from Foxo3-deficient mice.

Cell isolation and culture. Lymph node T cells were isolated by magnetic depletion of unwanted cells stained with a mix of biotinylated antibodies to B220, CD19, MHCII, DX5 and CD11b (all from eBioscience) and streptavidin-coupled microbeads (Miltenyi Biotec). B cells were isolated by magnetic positive selection from spleen cell suspensions stained with biotinylated antibody to CD19 and streptavidin-coupled microbeads. CD11c$^+$ and CD11b$^+$CD11c$^-$ cells were sorted on a FACSAria (BD Biosciences) from collagenase D–treated spleen (1 mg/ml for 30 min at 37 °C). Cell purity was routinely over 95%. For overnight culture, purified T cells were cultured at 5×10^6 cells/ml in complete RPMI medium (Gibco) supplemented with 5% FCS (Omega). For cell survival experiments, total lymph node cell suspensions were cleared from dead cells by density gradient centrifugation on Lympholyte-M (Cedarlane) and then cultured at 5×10^6 cells/ml in complete RPMI medium (Gibco) supplemented with 10% FCS (Omega). When indicated, cells were treated with 10 ng/ml recombinant mouse IL-7 (eBioscience).

Quantitative PCR. Total RNA was extracted from tissues or purified cell populations with TRIzol reagent (Invitrogen) according to the manufacturer's instructions, treated with DNase using a DNA-*free* kit (Ambion) and subjected to reverse transcription with SuperScript III reverse transcriptase and random hexamers (both from Invitrogen). cDNA was analyzed in duplicate by QPCR amplification using *Power* SYBR Green PCR Master mix (Applied Biosystems) supplemented with 30 nM of reference dye (Stratagene) on an Mx3005P system (Stratagene). Data were analyzed by comparative quantification with MxPro software. Primer sequences and PCR conditions are shown in **Supplementary Table 2** online.

Chromatin immunoprecipitation. Chromatin immunoprecipitation assays were done using a chromatin immunoprecipitation kit (17-295; Upstate Biotechnology) according to the manufacturer's instructions, with minor modifications. Briefly, 10×10^6 to 15×10^6 cells were fixed with 1% formaldehyde in PBS for 10 min at 25 °C with agitation. Fixed cells were immediately lysed with SDS lysis buffer for 10 min at 4 °C and sonicated with a digital Sonifier 250 (six 10-s pulses at 20% amplitude; Branson Ultrasonics). Lysates were diluted ten-fold, precleared for 2 h at 4 °C with salmon sperm DNA–protein A agarose, divided into two equal fractions and incubated overnight at 4 °C with 7.5 µg of either antibody to the transcription factor FKHR (H-128; Santa Cruz Biotechnology) or rabbit IgG. Protein-DNA immune complexes were then collected with protein A agarose beads, washed, eluted from the beads and incubated with NaCl (200 mM final concentration) for 4 h at 65 °C to reverse cross-links. After treatment with proteinase K, DNA was extracted with phenol-chloroform, precipitated with 100% ethanol for 2 h at –20 °C, washed and resuspended in Tris-EDTA buffer. Immunoprecipitates and input fraction were analyzed in duplicate by QPCR (**Supplementary Table 3** online).

Mixed bone marrow chimeras. T cell–depleted bone marrow cells from CD45.1$^+$CD45.2$^+$ *Foxo1*$^{f/f}$*-Cd4Cre* and CD45.1$^+$ *Foxo1*$^{f/f}$ littermates were mixed at a 1:1 ratio and injected intravenously into lethally irradiated CD45.2$^+$ C57BL/6 mice. Mice were killed and analyzed 8 weeks after reconstitution.

Statistics. Unpaired two-tailed Student *t* tests were used for statistical analysis, with GraphPad Prism software.

Accession codes. UCSD-Nature Signaling Gateway (http://www.signaling-gateway.org): A000944, A001417, A000630 and A001267.

Note: Supplementary information is available on the Nature Immunology website.

ACKNOWLEDGMENTS

We thank W. D'Souza and M. McGargill for discussions and assistance with adoptive transfer experiments; T. Ludwig (Columbia University) for *ERCre* mice; C. Murre (University of California at San Diego) for *PTEN*$^{f/f}$*-ERCre* mice; H. Cheroutre (La Jolla Institute for Allergy and Immunology) for *Il7r*$^{-/-}$ mice; S. Kaech for identifying the evolutionarily conserved regions of *Il7r*; and A. Goldrath and P. Marrack for critical reading of the manuscript. Supported by funds made available by the University of California, San Diego Division of Biological Sciences.

AUTHOR CONTRIBUTIONS

Y.M.K. designed and conducted all of the experiments, in collaboration with D.R.B., R.T. and A.S.D. The breeding and initial characterization of the *Foxo1; Cd4Cre* mice were carried out by D.R.B. and R.T. Mice with a *lox*P-targeted *Foxo1* locus were produced by D.H.C. and R.A.D. S.M.H. initiated the project with R.A.D. and supervised the experimentation. Y.M.K. and S.M.H. wrote the manuscript with contributions from the other authors.

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