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T cells require *Foxo1* to populate the peripheral lymphoid organs

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Summary

Forkhead (Fox) transcription factors play critical roles in leukocyte homeostasis. To study further the immunological functions of *Foxo1*, we generated mice that selectively lack *Foxo1* in T cells (*Foxo1* cKO). Although thymocyte development appeared relatively normal, *Foxo1* cKO mice harbored significantly increased percentages of mature single positive T cells in the thymus as compared to wild type (WT) mice, yet possessed smaller lymph nodes and spleens that contained fewer T cells. *Foxo1* cKO T cells were not more prone to apoptosis, but instead were characterized by a CD62L^{lo} CCR7^{lo} CD44^{hi} surface phenotype, a poorly populated lymphoid compartment in the periphery, and were relatively refractory to TCR stimulation, all of which were associated with reduced expression of *Sell*, *Klf2*, *Ccr7* and *Slpr1*. Thus, *Foxo1* is critical for naïve T cells to populate the peripheral lymphoid organs by coordinating a molecular program that maintains homeostasis and regulates trafficking.

Keywords

cell homing; homeostasis; knock out mice; lymph nodes; T cells

Introduction

For effective immunity, the naïve T cell population must be diverse enough such that a sufficient number of T cells will be specific for any pathogen that may be encountered, yet not unnecessarily drain an organism's energy resources [1]. This is accomplished by maintaining a pool of naïve T cells in a quiescent, non-dividing state. Naïve T cells circulate between the lymph nodes and spleen, where in the context of an infection, dendritic cells present peptides derived from the pathogen. Only a small number of T cells will have the appropriate T cell receptor to respond to this signal, but those that do will undergo rapid proliferation, acquire pathogen-specific effector functions, and efficiently battle the pathogen at the site of infection [2]. The lifespan of most effector T cells does not exceed a few weeks, but a small population of memory T cells can be detected many years after the infection [3].

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

The size of the peripheral T cell pool is determined by the rate of T cell egress from the thymus and the regulation of peripheral T cell survival and proliferation. T cell migration out of the thymus is dependent upon a chemotactic response to sphingosine-1-phosphate (S1p), mediated through S1p receptor-1 (S1pr1) [4]. Peripheral T cell homeostasis is thought to be controlled by signals received through the cytokine receptor common γ -chain (γ_c) and the T cell receptor (TCR). The survival of naïve T cells is ensured by contact with self peptide-MHC complexes (pMHC) and IL-7, a cytokine that signals through the cytokine receptor γ_c [3]. Competition for these ligands controls the size of the naïve T cell pool [3]. Accordingly, if the size of the naïve T cell pool is reduced, as in lymphopenic conditions, naïve T cells undergo proliferation and acquire a memory T cell phenotype [5]. Memory T cells are also generated during infectious responses as well as homeostatic proliferation driven by self-pMHC and commensal antigens. Although the mechanisms controlling the homeostasis of this heterogeneous group of memory T cells varies, most require exposure to IL-7 and IL-15, but not MHC. IL-7 and IL-15 each contribute to the survival and cell division capabilities of CD4+ memory T cells, whereas IL-15 is sufficient to control the homeostatic proliferation and survival of most CD8+ memory T cells. A notable exception are CD8+ memory T cells generated in response to pathogens, which require IL-7 for their survival [5].

The ability of T cells to circulate through peripheral lymphoid organs is important for their survival and potential to encounter cognate antigen within the appropriate context. Whereas access to the spleen appears relatively uncomplicated, the trafficking of T cells into lymph nodes is tightly regulated and occurs in three stages. First, T cell velocity through the blood vessel is slowed via weak interactions between vascular ligands and the T cell expressed receptors: Selectin, lymphocyte (Sell, CD62L) and Integrin β_2 (Itgb2/LFA-1) [6]. Chemokines, such as Chemokine (C-C motif) ligand 19 and 21 (CCL19 and CCL21), then signal through their common T cell receptor, chemokine (CC motif) receptor 7 (CCR7), to induce firm adhesion and promote extravasation through specialized high-endothelial venules [6]. Once across the endothelial barrier, the precise localization of cells to the T cell zone is controlled through CCR7-dependent chemotaxis to a CCL19 gradient [7]. The expression of CCR7 and CD62L are critical for the proper homing of naïve T cells and appears to be controlled, at least in part, by *Krüppel-like factor 2* (*Klf2*) [8].

Members of the forkhead o (Foxo) transcription factor subfamily share a winged helix DNA binding domain and influence many biological processes, including development, aging, cancer, metabolism, and immune regulation, typically by regulating genes involved in cell survival, death, proliferation, and the stress response [9]. Foxo proteins are transcriptionally active during cellular quiescence, while the presence of growth factors triggers their phosphatidylinositol-3-OH kinase (PI(3)K)-dependent export to the cytoplasm [10]. Conversely, oxidative stress and growth factor withdrawal results in the return of Foxo proteins to the nucleus [11,12].

The role of Foxo subfamily members in the immune system has begun to be elucidated. Mice deficient for *Foxo3a* experience a systemic and spontaneous autoimmune syndrome due to hyperactive NF- κ B signaling in T cells [13]. In Jurkat T cells, *Foxo1* overexpression suppresses proliferation [14], while over-expressing a constitutively active *Foxo3a* induces apoptosis [15]. Additionally, exposure of T cell lines to IL-2 results in the inhibition of Foxo family members, while IL-2 withdrawal activates Foxo proteins [16]. Together, these observations suggest that Foxo subfamily members regulate T cell quiescence and homeostasis.

Recently, it was reported that conditional deletion of *Foxo1* in T cells alters naïve T cell homing and survival as a result of dysregulated expression of *Bcl2*, *Sell*, *Ccr7*, and

interleukin 7 receptor α [17]. In this study, we confirm a critical role for *Foxo1* in the establishment of the peripheral naïve T cell pool – but surprisingly, in our studies, T cell-specific deficiency of *Foxo1* results in dramatically diminished absolute numbers of CD4+ and CD8+ T cells in the periphery, along with increased percentages of SP mature thymocytes. Moreover, *Foxo1*-deficient T cells resembled memory T cells and expressed lower levels of *Sell*, *Slpr1*, *Klf2*, and *Ccr7*. Thus, our data indicate that *Foxo1* not only regulates T cell trafficking and survival [17], but is also critical for T cell population of the peripheral lymphoid organs by regulating thymic egress and/or homeostatic proliferation. Together, these studies implicate *Foxo1* in multiple aspects of naïve T cell homeostasis.

Results

Efficiency of *Foxo1* conditional knockout

Foxo1^{flx/flx} mice were crossed to transgenic mice expressing cre recombinase under the proximal *Lck* promoter to obtain *Foxo1*^{flx/flx} *Lck.cre*+ T cell-specific conditional knockout mice (cKO) and *Foxo1*^{flx/flx} *Lck.cre*- wild type (WT) controls. PCR was used to assess the genomic configuration of the *Foxo1* gene in sorted double negative (DN), double positive (DP), CD4 single positive (SP), and CD8 SP thymocytes isolated from *Foxo1* cKO or WT mice. Genomic DNA obtained from DN thymocytes sorted from *Foxo1* cKO mice yielded PCR products specific for both the intact floxed and deleted *Foxo1* allele (data not shown). Only the PCR product specific to the floxed *Foxo1* allele was obtained from all WT thymocyte samples (Figure 1B). In the more mature thymocyte subpopulations (DP, CD4 SP, and CD8 SP) isolated from *Foxo1* cKO mice, only the PCR product specific to the deleted *Foxo1* allele was detected (Figure 1B). Taqman RT-PCR analysis revealed dramatic reductions of *Foxo1* mRNA in DP, CD4+, and CD8+ cKO thymocytes as compared to WT thymocytes (Figure 1C). Moreover, *Foxo1* protein was undetectable by western blot in *Foxo1* cKO thymocyte lysate, but present in WT thymocyte lysate (Figure 1D). Thus, analyses of genomic DNA, mRNA, and protein samples confirm that *Foxo1* is efficiently deleted from *Foxo1* cKO thymocytes after the DN stage of thymocyte development.

Analysis of thymic and peripheral T cell populations

The absolute numbers of thymocytes isolated from WT and *Foxo1* cKO mice were similar, averaging almost 75 million cells in each group (data not shown). The percentages of DN thymocytes were also equivalent in *Foxo1* cKO and WT mice, but *Foxo1* cKO mice possessed a slightly lower percentage of DP thymocytes, with concomitant increases in CD4 and CD8 SP thymocyte populations (Figure 2A). Interestingly, the SP thymocytes in *Foxo1* cKO mice expressed high levels of Qa-2, but low levels of CD69, a phenotype associated with the most mature SP thymocytes (Figure 2B). *Foxo1* cKO mice also demonstrated increased percentages of CD69+ Qa-2+ SP thymocytes (Figure 2B).

Surprisingly, the spleens and lymph nodes of *Foxo1* cKO mice were smaller and contained less than half the absolute numbers of T cells found in their WT counterparts (Figure 2C). The percentage of CD8+ T cells was most dramatically decreased in both organs isolated from *Foxo1* cKO mice (Figure 2D). Thus the CD4/CD8 ratio of peripheral T cells in *Foxo1* cKO was skewed in favor of CD4+ T cells (3.6 in cKO mice, as compared to ~2.0 in WT mice).

Reduced numbers of T cells in the periphery of cKO mice indicated that *Foxo1*-deleted T cells were less efficient in populating the peripheral lymphoid organs and suggested that peripheral T cells in cKO mice may actually be ‘WT’ cells, representing T cells that escaped cre-mediated *Foxo1* deletion in the thymus. Indeed, *Foxo1* cKO T cells sorted from the spleen, lymph nodes, and blood yielded PCR products specific to both the intact floxed and

deleted *Foxo1* alleles (Figure 2E). Furthermore, bulk T cell populations isolated from the spleen of WT and cKO mice exhibited similar levels of *Foxo1* mRNA (Figure 2F). Thus, although *Foxo1* is efficiently deleted from T cells during thymocyte development in cKO mice, such T cells fail to populate the lymphoid periphery as efficiently as WT cells; T cells that have escaped cre-mediated *Foxo1* deletion out-compete them and in turn comprise the majority of the peripheral T cell compartment.

Apoptotic and proliferative capacities of WT and *Foxo1* cKO thymocytes

Ex vivo percentages of *Foxo1* cKO and WT thymocytes undergoing apoptosis were relatively low and similar for both groups, but twice as many CD4⁺ WT thymocytes were positive for apoptosis markers as compared to CD4⁺ cKO thymocytes (Figure 3A). *In vitro* rates of apoptosis in response to T cell stimulation did not significantly differ between WT and cKO SP thymocytes under any of the conditions tested (Supplementary Figure 1A), and there were no differences in the mRNA levels of the pro- or anti-apoptotic genes (*Fas ligand* (*Fas*) and *Bcl2-like 11* (*Bcl2l1* or *Bim*) *B-cell leukemia/lymphoma 2* (*Bcl2*) and *Bcl2-like 1* (*Bcl2l1*)), analyzed in sorted thymocyte populations from WT and cKO mice (Supplementary Figure 1B).

In contrast, SP thymocytes isolated from WT mice proliferated almost twice as well as cKO cells in response to either anti-CD3 or anti-CD3 plus anti-CD28 stimulation conditions (Figure 3B): about 21% of CD4⁺ T cells and 29% of CD8⁺ T cells isolated from cKO thymus proliferated in response to plate-bound anti-CD3 as compared to 33% of CD4⁺ T cells and 46% of CD8⁺ T cells isolated from WT mice. Similarly, approximately 30% of CD4⁺ and CD8⁺ cKO T cells proliferated in response to anti-CD3 + anti-CD28 stimulation as compared to almost 60% of CD4⁺ and CD8⁺ WT T cells (Figure 3B). WT and cKO T cells underwent a similar number of cellular divisions, however, a greater percentage of WT T cells completed a larger number of divisions resulting in an overall higher percentage of divided cells. Differences in the percentages of divided T cells are unlikely related to lack of accumulation of cKO cells, since rates of apoptosis were not greater in cKO T cells (Figure 3A). Thus, although *Foxo1* cKO SP thymocytes appear to possess a grossly intact apoptotic response, they possess a modestly reduced proliferative response to TCR stimulation.

Foxo1 cKO T cells are CD62L^{lo}, CCR7^{lo}, and CD44⁺

We anticipated that altered genetic expression of *Foxo1* target genes involved in trafficking, homeostasis, and cell growth may further underlie the absence of *Foxo1*-deleted T cells in the periphery. Therefore, we measured the mRNA levels of several genes considered to be T cell specific *Foxo1* target genes including *Ccr7*, *Klf2*, *Sell*, *S1pr1*, *S1pr4*, *early growth response 1, 2, and 3* (*Egr1*, *Egr2*, and *Egr3*) in sorted thymocyte populations isolated from cKO and WT mice [18]. There were no differences in the mRNA levels of *S1pr4*, *Egr1*, *Egr2*, and *Egr3* (data not shown). However, CD4⁺ and CD8⁺ thymocytes from cKO mice demonstrated a trend toward reduced *Ccr7* and *Klf2* mRNA levels and exhibited significantly reduced levels of *S1pr1* and *Sell* mRNA (Figure 4A). Moreover, protein levels of CD62L and CCR7 were significantly reduced in cKO CD4⁺ and CD8⁺ thymocytes as compared to WT thymocytes (Figure 4B).

Since lower levels of CD62L are associated with either activated or memory T cell phenotypes, we assessed the levels of other activation and memory T cell markers on peripheral T cells isolated from WT and *Foxo1* cKO mice (Figure 5). Overall, CD4⁺ and CD8⁺ T cells isolated from the blood, spleen, and LN of cKO mice demonstrated reduced expression of CD62L and higher levels of CD44, but not CD25 (Figure 5 and 6B). A small portion of the CD62L^{lo} CD44⁺ CD25⁻ CD122⁻ T cells in the lymph nodes and spleens of cKO mice were also positive for CD69 or CD27, however, most T cells in cKO mice were

of the CD62L^{lo} CD44⁺ CD25⁻ CD122⁻ CD69⁻ CD27⁻ phenotype. About 20% of CD8⁺ T cells isolated from the periphery of cKO mice also expressed high levels of CD122, though CD122 expressing cells were not CD62L^{lo} or CD44^{hi}. Thus, cKO T cells are characterized by an unusual CD62L^{lo}CCR7^{lo}CD44⁺ phenotype, associated with reduced expression of the egress-relevant *S1pr1*.

Low levels of CD62L correlate with the deleted *Foxo1* allele and can be used to identify *Foxo1* cKO T cells in the periphery

Given the findings above, CD62L surface protein on CD4⁺ and CD8⁺ T cells may correlate with the genomic status of *Foxo1*—i.e. a CD62L^{lo} phenotype may correlate with the deleted *Foxo1* allele while a CD62L^{hi} phenotype may correlate with escapee cells containing intact *Foxo1* genomic DNA. Indeed, the PCR product specific to the floxed *Foxo1* allele was enriched in CD4⁺ and CD8⁺ T cells with high levels of CD62L, while the PCR product specific for the deleted *Foxo1* allele was enriched in CD4⁺ and CD8⁺ T cells with low levels of CD62L (Figure 6A).

We therefore used CD62L expression to ascertain the ability of *Foxo1*-deleted T cells to populate the lymphoid periphery. A significantly higher percentage of CD62L^{lo} T cells was present in the thymus, blood, and spleen of cKO mice than in WT mice (Figure 6B). Moreover, the percentage of CD62L^{lo} T cells was decreased in the blood and spleen as compared to the thymus, and not surprisingly, the percentage of CD62L^{lo} T cells was quite low in the lymph nodes of both WT and cKO mice, consistent with a known requirement for CD62L in lymph node entry by T cells [19]. Most of the CD4⁺ CD62L^{lo} *Foxo1*-deleted cells expressed high levels of CD44 in the spleen, LN, and blood (average ± standard deviation: 81±5%, 76±6%, 69±10%, respectively) (data not shown). Similarly, high percentages of CD4⁺ CD62L^{lo} WT cells were also CD44^{hi} (average ± standard deviation: 91±4%, spleen; 85±2%, LN; 64±7%, blood) (data not shown). These analyses indicate that *Foxo1*-deleted cells (CD62L^{lo}) can clearly egress from the thymus, although perhaps less efficiently than WT cells, yet nonetheless experience a significant disadvantage in populating the peripheral lymphoid compartments.

Discussion

We report that selective deletion of *Foxo1* from T cells resulted in increased percentages of mature single positive T cells in the thymus, with diminished proliferative capacity in response to T cell stimulation, and reduced absolute numbers of T cells in the peripheral lymphoid organs. Moreover, peripheral T cells in cKO mice resembled memory T cells, and were largely absent from the lymph nodes. Our data suggest that *Foxo1* regulation of *Sell*, *Klf2*, *Ccr7* and *S1pr1* contribute to the lack of peripheral T cells in *Foxo1* cKO mice likely by altering T cell homing, peripheral T cell survival, and homeostatic proliferation. In contrast to these findings, a recent study reported largely unaffected numbers of total CD4⁺ T cells, with reduced naïve but increased memory CD4⁺ T cells, and only a trend towards increased CD8 SP, but not CD4 SP thymocytes, in mice with T cell-specific deficiency of *Foxo1* [17]. The subtle differences in phenotype observed between the two groups may be related to the age and/or genetic background of the mice used in each study, as both variables have been observed to influence the onset and severity of lymphocyte abnormalities in other studies [20].

Although impaired homeostatic proliferation might contribute to the peripheral T cell lymphopenia in cKO mice, the modest proliferative impairment in response to TCR stimulation we observed in *Foxo1* deficient T cells seems disproportionate with the magnitude of T lymphopenia. Instead, one key mechanism by which *Foxo1* likely regulates the size of the peripheral T cell population is its known role in the regulation of *Sell*, the

gene encoding CD62L [17,18], as confirmed here. Diminished surface expression of CD62L protein in *Foxo1*-deficient T cells likely hinders their ability to compete with WT T cells in populating the secondary lymphoid organs, analogous to findings in *Sell* deficient mice [21]. Long-term inhibition of lymph node entry by lymphocytes significantly reduces the size of the total peripheral T cell pool, likely by precluding access to local survival factors [22]. Thus, lack of CD62L surface protein expression on *Foxo1*-deficient T cells probably contributes to the deficit of peripheral T cells by eliminating access to the peripheral lymph nodes and their exposure to survival factors. The paucity of peripheral T cells is likely further exacerbated by reduced expression of *IL-7ra* in *Foxo1*-deficient T cells [17].

In addition, impaired thymic egress may also contribute to cKO T cell lymphopenia. Although *Foxo1* cKO T cells were capable of thymic egress, *Foxo1* cKO thymi possessed increased percentages of mature SP thymocytes, which may reflect accumulation due to decreased rates of thymic egress. Supportively, *Slpr1* and *Klf2* mRNA levels were reduced in *Foxo1* cKO SP thymocytes and mice with genetically modified expression of *Slpr1* and *Klf2* demonstrate irregular thymic egress characterized by the accumulation of mature SP T cells in the thymus [4,8]. Moreover, *Foxo1* transcriptional activity is regulated by PI(3)K, and mice lacking p110 γ , the catalytic subunit of PI(3)K class IB, demonstrate increased percentages of SP thymocytes as a result of diminished thymic egress—whereas mice transgenic for an activating mutation of the PI(3)K class IA regulatory subunit (p65^{PI(3)K}) exhibit increased rates of thymic egress [23]. Since PI(3)K regulates the transcriptional activity of *Foxo1*, it is possible that the alterations in thymic egress in p110 γ KO and p65^{PI(3)K} transgenic mice are related to changes in *Foxo1* activity.

The increased prevalence of peripheral T cells with memory cell phenotypes in *Foxo1* cKO mice was an unexpected finding. Considering that the peripheral lymphoid compartment in *Foxo1* cKO mice is somewhat lymphopenic, increases in lymphopenia-induced homeostatic proliferation, after which T cells irreversibly acquire memory T cell surface markers [3], may explain this phenomenon. Alternatively, memory T cells have been shown to exist in animals not exposed to antigen [24]. Although it is unclear how such memory T cells develop, it is possible that *Foxo1* regulates the development, survival, or apoptosis of them. Accordingly, dysregulation of *Foxo1* in older p65^{PI(3)K} transgenic mice is associated with a lymphoproliferative disorder due to enhanced memory T cell survival [25]. Finally, *Foxo1* may regulate a host of genes associated with the naïve T cell state, including *Sell*. In its absence, T cells instead assume the phenotype of activated or memory T cells.

Similar phenotypes have been observed in mice with T cell specific deficiencies of *Foxo1*, *Klf2*, and *Slpr1*, suggesting involvement of all three molecules in a common pathway. For example, like *Foxo1* cKO mice, fetal liver chimeras generated with *Slpr1* deficient donors and mice with T cell-specific *Klf2* deficiency demonstrated reduced numbers of T cells in the peripheral lymphoid organs along with alterations in thymocyte subpopulations as described above [4,8]. However, naïve *Klf2* deficient T cells also inappropriately expressed many inflammatory chemokine receptors, resulting in their trafficking to non-lymphoid peripheral tissues [26], a phenomenon not observed in either *Foxo1* cKO mice (our unpublished observations) or *Slpr1* KO fetal liver chimeras. Moreover, we observed decreased levels of *Klf2* mRNA in *Foxo1* cKO T cells and *Foxo1* has been shown to bind the *Klf2* promoter in an EMSA [18]. It is possible that diminished levels of *Klf2* in *Foxo1* cKO mice alone could explain the down-regulation of *Sell* and *Slpr1*, since both are known targets of *Klf2*. However, *Klf2* mRNA levels are only modestly reduced, while *Sell* and *Slpr1* mRNA levels are more dramatically reduced, suggesting that *Klf2* and *Foxo1* may cooperate in the transactivation of common target genes. In keeping with this possibility, the proximal noncoding region of the CD62L gene does not contain a *Foxo1* consensus DNA-binding sequence (our unpublished data). Thus, *Foxo1* may regulate T cell homeostasis and

population of peripheral lymphoid organs via a coordinate regulation of *Sell*, *Klf2*, *Ccr7*, and *S1pr1*. Future studies will be of interest to understand further the molecular mechanisms by which *Foxo1* regulates such a genetic program.

Materials and Methods

Mice

LoxP sites were introduced 2518 bp upstream and 1756 bp downstream of the start codon in the first exon of *Foxo1* in embryonic stem (ES) cells by the targeting vector shown in Figure 1a. Thus a 4775 bp fragment was floxed. Neomycin resistant ES cell clones were screened by PCR and the targeted ES cell clones were aggregated with CD-1 embryos to generate chimeras. Germline chimeras were crossed with Rosa26-*Flp* (129S4/SvJaeSor-Gt(ROSA)26Sortm1(FLP1)Dym/J) mice to remove the PGKNeo cassette and generate *Foxo1^{flox/+}* mice. Conditional deletion of *Foxo1* in cells of T lymphocyte lineage was achieved in *Foxo1^{flox/flox}Lck-Cre^{+/-}* mice by breeding *Foxo1^{flox/flox}* mice with *Lck.cre* transgenic mice (C57BL/6NTac-TgN(*Lck-Cre*)). All mice were confirmed to be of C57BL/6 lineage (>99%) based on microsatellite mapping (data not shown). Rosa26-*Flp* mice were purchased from The Jackson Laboratory and *Lck-Cre* mice were purchased from Taconic Farms, Inc. All mice were housed under specific pathogen free conditions at either the University of Connecticut, Charles River Laboratories, or Roche Palo Alto and studied according to Institutional Animal Care and Use Committee-approved guidelines and protocols. Mice used in experiments were 2-7 months of age. Age-matched littermate controls were used in all experiments.

Flow cytometry reagents

Reagents used for flow cytometry experiments include: anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-CD62L (MEL-14), anti-CCR7 (EBI-1) (eBiosciences, San Diego, CA), anti-CD69 (H1.2F3), anti-CD44 (IM7) (eBiosciences), anti-CD25 (PC61), Annexin V, DAPI (Sigma, St. Louis, MO), anti-CD27 (LG.3A10), anti-Qa2 (1-1-2), and anti-CD122 (5H4). All antibodies listed were purchased from BD Biosciences (San Jose, CA), except where noted above.

Flow cytometry and cell sorting

Splenocytes were cleared of red blood cells by osmotic lysis and then incubated with an Fc-receptor blocking reagent (2.4G2, BD Pharmingen, San Diego, CA) and various combinations of staining antibodies. Data were obtained on a LSR II (BD Biosciences) and analyzed with FlowJo software (Treestar, Inc., Ashland, OR). The indicated cell populations were sorted with a FACSAriaTM cell sorter (BD Biosciences), obtaining at least 95% purity.

PCR

Genomic DNA was isolated using the DNeasy 96 well kit (Qiagen, Valencia, CA). PCR primer b, specific to the floxed allele (5'-AATCAATGCCCCCAATACAA), primer c to the deleted allele (5'-CACACCTCGAGAGGGACTTC), and a common primer a (5'-GACCCTCCACATTTACTTCC) were utilized to detect each *Foxo1* allele (Figure 1a). PCR amplicons are 346 bp, 431 bp and 261 bp for wildtype, floxed and deleted alleles respectively.

Taqman RT-PCR

RNA was isolated from sorted cells using the RNeasy 96 well kit (Qiagen, Valencia, CA) and then reverse transcribed using Amplitaq Gold (Applied Biosystems, Foster City, CA) according to the manufacturer's recommendations. cDNA was subjected to Taqman

quantitative PCR in triplicate on a ABI PRISM 7900 HT Sequence Detection System using TaqManUniversal PCR Master Mix (Applied Biosystems). mRNA levels of each gene were normalized to mRNA levels of *hypoxanthine guanine phosphoribosyl transferase 1 (Hprt1)* to ensure equal RNA input. All primer/probe sets were obtained as inventoried TaqMan® Gene Expression Assays from Applied Biosystems.

Immunoblots

Cell lysates were obtained from sorted cells using buffers supplied with the NucBuster™ Protein Extraction Kit (Novagen, San Diego, CA). The total protein concentration was measured using the Bio-Rad protein assay kit (Biorad, Hercules, CA). Ten micrograms of total protein was subjected to 10% SDS-PAGE and blotted onto a polyvinylidene fluoride membrane. The membrane was blocked with 5% non-fat dry milk in PBS and incubated with anti-Foxo1 (L27) or anti-β actin (13E5) monoclonal antibody (1:1000) (Cell Signaling Technologies, Danvers, MA). The blots were then incubated with HRP-conjugated anti-rabbit antibody (1:3000) (Cell Signaling Technologies) at room temperature for 2 h. The labeled bands were detected by chemiluminescence using Amersham ECL™ Western Blotting Detection Reagents (GE Healthcare, Piscataway, NJ).

Apoptosis and proliferation assays

Thymocytes were labeled with 2.5 μM CFSE (Invitrogen) and one million cells were cultured on anti-CD3 (145–2C11, BD Biosciences) coated 96-well flat-bottomed plates ±3 μg/ml of anti-CD28 (37.51, BD Biosciences) for 72 hours. For the apoptosis assay, unlabelled thymocytes were cultured on anti-CD3 coated 96-well flat-bottomed plates. After 18 hours, cells were stained with anti-CD4, anti-CD8, Annexin V, and DAPI and analyzed by flow cytometry.

Statistical analysis

The statistical significance of differences between groups of mice was determined using a nonparametric Mann-Whitney *U* test, Student's *t* test, or unpaired *t* test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

Foxo	<i>forkhead o</i>
SLp	<i>sphingosine-1-phosphate</i>
γ_c	cytokine receptor common γ-chain
PI(3)K	phosphatidylinositol-3-OH kinase
DN	double negative
DP	double positive
SP	single positive

cKO	<i>Foxo1^{flox/flox} Lck.cre+</i> conditional knock out mice
<i>Fasl</i>	<i>Fas ligand</i>
<i>BCL2l1l</i>	<i>Bcl2-like 11</i>
<i>Bcl2</i>	<i>B-cell leukemia/lymphoma 2</i>
<i>Bcl2l1</i>	<i>Bcl2-like 1</i>
<i>Ccr7</i>	<i>Chemokine (C-C motif) ligand</i>
<i>CCL</i>	<i>chemokine (C-C motif) receptor 7</i>
<i>Klf2</i>	<i>Krüppel-like factor 2</i>
<i>Sell</i>	<i>selectin, lymphocyte</i>
<i>Egr</i>	<i>early growth response</i>
<i>hprt1</i>	<i>hypoxanthine guanine phosphoribosyl transferase 1</i>

References

1. Fox CJ, Hammerman PS, Thompson CB. Fuel feeds function: energy metabolism and the T-cell response. *Nat Rev Immunol*. 2005; 5:844–852. [PubMed: 16239903]
2. Marelli-Berg FM, Okkenhaug K, Mirenda V. A two-signal model for T cell trafficking. *Trends Immunol*. 2007; 28:267–273. [PubMed: 17481953]
3. Surh CD, Sprent J. Homeostasis of naive and memory T cells. *Immunity*. 2008; 29:848–862. [PubMed: 19100699]
4. Matloubian M, Lo CG, Cinamon G, Lesneski MJ, Xu Y, Brinkmann V, Allende ML, Proia RL, Cyster JG. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature*. 2004; 427:355–360. [PubMed: 14737169]
5. Sprent J, Cho JH, Boyman O, Surh CD. T cell homeostasis. *Immunol Cell Biol*. 2008; 86:312–319. [PubMed: 18362947]
6. Ebert LM, Schaerli P, Moser B. Chemokine-mediated control of T cell traffic in lymphoid and peripheral tissues. *Mol Immunol*. 2005; 42:799–809. [PubMed: 15829268]
7. Sprent J, Surh CD. T cell memory. *Annu Rev Immunol*. 2002; 20:551–579. [PubMed: 11861612]
8. Carlson CM, Endrizzi BT, Wu J, Ding X, Weinreich MA, Walsh ER, Wani MA, Lingrel JB, Hogquist KA, Jameson SC. Kruppel-like factor 2 regulates thymocyte and T-cell migration. *Nature*. 2006; 442:299–302. [PubMed: 16855590]
9. Jonsson H, Peng SL. Forkhead transcription factors in immunology. *Cell Mol Life Sci*. 2005; 62:397–409. [PubMed: 15719167]
10. Peng SL. Foxo in the immune system. *Oncogene*. 2008; 27:2337–2344. [PubMed: 18391975]
11. Tothova Z, Kolipara R, Huntly BJ, Lee BH, Castrillon DH, Cullen DE, McDowell EP, Lazo-Kallanian S, Williams IR, Sears C, Armstrong SA, Passegue E, DePinho RA, Gilliland DG. FoxOs are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress. *Cell*. 2007; 128:325–339. [PubMed: 17254970]
12. You H, Pellegrini M, Tsuchihara K, Yamamoto K, Hacker G, Erlacher M, Villunger A, Mak TW. FOXO3a-dependent regulation of Puma in response to cytokine/growth factor withdrawal. *J Exp Med*. 2006; 203:1657–1663. [PubMed: 16801400]
13. Lin L, Hron JD, Peng SL. Regulation of NF-kappaB, Th activation, and autoinflammation by the forkhead transcription factor Foxo3a. *Immunity*. 2004; 21 :203–213. [PubMed: 15308101]
14. Medema RH, Kops GJ, Bos JL, Burgering BM. AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. *Nature*. 2000; 404:782–787. [PubMed: 10783894]

15. Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell*. 1999; 96:857–868. [PubMed: 10102273]
16. Stahl M, Dijkers PF, Kops GJ, Lens SM, Coffey PJ, Burgering BM, Medema RH. The forkhead transcription factor FoxO regulates transcription of p27Kip1 and Bim in response to IL-2. *J Immunol*. 2002; 168:5024–5031. [PubMed: 11994454]
17. Kerdes Y, 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. *Nat Immunol*. 2009; 10:176–184. [PubMed: 19136962]
18. Fabre S, Carrette F, Chen J, Lang V, Semichon M, Denoyelle C, Lazar V, Cagnard N, Dubart-Kupperschmitt A, Mangeney M, Fruman DA, Bismuth G. FOXO1 regulates L-Selectin and a network of human T cell homing molecules downstream of phosphatidylinositol 3-kinase. *J Immunol*. 2008; 181:2980–2989. [PubMed: 18713968]
19. Bradley LM, Watson SR, Swain SL. Entry of naive CD4 T cells into peripheral lymph nodes requires L-selectin. *J Exp Med*. 1994; 180:2401–2406. [PubMed: 7525854]
20. Zhu JW, Brdicka T, Katsumoto TR, Lin J, Weiss A. Structurally distinct phosphatases CD45 and CD148 both regulate B cell and macrophage immunoreceptor signaling. *Immunity*. 2008; 28:183–196. [PubMed: 18249142]
21. Arbones ML, Ord DC, Ley K, Ratech H, Maynard-Curry C, Otten G, Capon DJ, Tedder TF. Lymphocyte homing and leukocyte rolling and migration are impaired in L-selectin-deficient mice. *Immunity*. 1994; 1:247–260. [PubMed: 7534203]
22. Link A, Vogt TK, Favre S, Britschgi MR, Acha-Orbea H, Hinz B, Cyster JG, Luther SA. Fibroblastic reticular cells in lymph nodes regulate the homeostasis of naive T cells. *Nat Immunol*. 2007; 8:1255–1265. [PubMed: 17893676]
23. Rodriguez-Borlado L, Barber DF, Hernandez C, Rodriguez-Marcos MA, Sanchez A, Hirsch E, Wymann M, Martinez AC, Carrera AC. Phosphatidylinositol 3-kinase regulates the CD4/CD8 T cell differentiation ratio. *J Immunol*. 2003; 170:4475–4482. [PubMed: 12707323]
24. Haluszczak C, Akue AD, Hamilton SE, Johnson LD, Pujanauski L, Teodorovic L, Jameson SC, Kedl RM. The antigen-specific CD8+ T cell repertoire in unimmunized mice includes memory phenotype cells bearing markers of homeostatic expansion. *J Exp Med*. 2009; 206:435–448. [PubMed: 19188498]
25. Borlado LR, Redondo C, Alvarez B, Jimenez C, Criado LM, Flores J, Marcos MA, Martinez AC, Balomenos D, Carrera AC. Increased phosphoinositide 3-kinase activity induces a lymphoproliferative disorder and contributes to tumor generation in vivo. *Faseb J*. 2000; 14:895–903. [PubMed: 10783143]
26. Sebзда E, Zou Z, Lee JS, Wang T, Kahn ML. Transcription factor KLF2 regulates the migration of naive T cells by restricting chemokine receptor expression patterns. *Nat Immunol*. 2008; 9:292–300. [PubMed: 18246069]

Figure 1. Efficiency of *Foxo1* deletion in thymocytes obtained from *Foxo1* cKO mice

(A) *Foxo1* genomic loci containing wild-type (WT), loxP sequence-flanked (floxed) and first exon-deleted (del) alleles are represented schematically. Genotyping PCR primers (a, b, and c) used and their relative placements along the *Foxo1* loci are also shown. (B) The genomic DNA of sorted DP, CD4 SP, and CD8 SP thymocytes from cKO or WT mice was subjected to PCR to assess the genomic status of the *Foxo1* gene. The PCR products obtained from the intact floxed and del conditions are indicated by arrows. (C) mRNA obtained from sorted thymocytes was analyzed by Taqman RT-PCR for *Foxo1* and normalized to *Hprt1* mRNA levels. The mean and standard deviation are shown. (D) Western blot of lysates obtained from DP thymocytes probed with anti-FoxO1 and anti-actin antibodies. Data are representative of three separate experiments and each DNA, RNA, or lysate preparation was obtained from a pool of at least three mice. * $p < 0.05$, Mann-Whitney t test.

Figure 2. Analysis of thymic and peripheral T cell populations in WT and cKO mice
 (A) Percentages of the indicated thymocyte subpopulations were determined by flow cytometry. (B) The percentage of CD4 SP and CD8 SP thymocytes expressing Qa-2 and/or CD69 was determined by flow cytometry. (C) CD4⁺ and CD8⁺ T cells in the spleens and LN of WT and cKO mice, as determined by flow cytometry (mean+SD) (n=5 for each group). (D) The percentages of CD8⁺ T cells in the spleen and LN of WT and cKO mice. (E) Genomic DNA isolated from CD4⁺ and CD8⁺ T cells sorted from the spleen, LN, and blood of WT and cKO mice was subjected to PCR to assess the genomic status of the *Foxo1* gene. (F) mRNA was isolated from sorted splenic CD4⁺ and CD8⁺ T cells from WT and cKO mice and levels of *Foxo1* mRNA were assessed by Taqman RT-PCR and normalized to mRNA levels of *Hprt1*. Data show mean + SD (n=3 for each group). Data are representative of three independent experiments and each DNA or RNA preparation was obtained from a pool of at least three mice. *p<0.05, Mann-Whitney *t* test.

Figure 3. Assessing the apoptotic and proliferative capacities of WT and cKO thymocytes
 (A) The rate of *ex vivo* apoptosis was measured by flow cytometry in thymocytes isolated from WT and cKO mice and stained with DAPI and Annexin V. * $p < 0.05$, Mann-Whitney t test. (B) Thymocytes isolated from WT and cKO mice were labeled with CFSE and cultured with plate-bound anti-CD3 $\pm$ anti-CD28. After 72 hours, CFSE dilution was assessed by flow cytometry. Values on plots indicate mean percentages of CFSE^{low} cells $\pm$ SEM (n=3, in each group). * $p < 0.05$, paired t test. Data are representative of three separate experiments.

Figure 4. Analysis of FoxO1 target genes in WT and cKO thymocytes

(A) The mRNA levels of *Ccr7*, *Klf2*, *S1pr1*, and *Sell* were assessed in the indicated sorted thymocyte populations by Taqman RT-PCR and normalized to *Hprt1* mRNA levels. Data show mean + SD (n=3, for each group). *p<0.05, unpaired *t* test. (B) Expression levels of CD62L and CCR7 were analyzed by flow cytometry on thymocytes isolated from WT (values in right corner of histograms) and cKO mice (values in left corner of histograms). The values on CD62L histograms represent mean fluorescent intensities (MFI), while values on CCR7 histograms represent the percent of cells within the CCR7+ gate. Data are representative of two separate experiments with 3–5 mice per group in each experiment.

Figure 5. Phenotype of peripheral T cells in WT and cKO mice

Expression levels of CD69, CD44, CD25, CD122, and CD27 were analyzed by flow cytometry on cells isolated from the blood, LN, and spleen of WT and cKO mice. Values on all plots (except for the CD27 LN plots) indicate mean percentages of cells within the indicated gates $\pm$ SEM (n=5). Values on the CD27 LN plots represent mean fluorescence intensities $\pm$ SEM (n=5). cKO percentages are underlined. *p<0.05, Mann-Whitney *t* test. Data are representative of at two separate experiments.

Figure 6. Tracking *Foxo1*-deleted T cells in the peripheral lymphoid compartments of cKO mice
 (A) Genomic DNA of CD4⁺ and CD8⁺ T cells sorted by levels of CD62L protein (CD62L^{hi} vs. ^{lo}) obtained from the spleens of WT and cKO mice was subjected to PCR to assess the genomic condition of the *Foxo1* gene. PCR products resulting from the intact floxed and deleted alleles are indicated. (B) Expression levels of CD62L were analyzed by flow cytometry on cells isolated from the thymi, blood, LN, and spleens of WT and cKO mice. Values on plots represent the percentages of CD62L^{lo} cells $\pm$ SEM (n=5). cKO percentages are underlined. *p<0.05, Mann-Whitney *t* test. Data are representative of three separate experiments.