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An Essential Role of the Forkhead-Box Transcription Factor Foxo1 in Control of T Cell Homeostasis and Tolerance

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SUMMARY

Members of the Forkhead box O (Foxo) family of transcription factors are key regulators of cellular responses, but their function in the immune system remains incompletely understood. Here we showed that T cell-specific deletion of *Foxo1* gene in mice led to spontaneous T cell activation, effector T cell differentiation, autoantibody production, and the induction of inflammatory bowel disease in a transfer model. In addition, Foxo1 was critical for the maintenance of naive T cells in the peripheral lymphoid organs. Transcriptome analyses of T cells identified Foxo1-regulated genes encoding, among others, cell-surface molecules, signaling proteins, and nuclear factors that control gene expression. Functional studies validated interleukin-7 receptor-α as a Foxo1 target gene essential for Foxo1 maintenance of naive T cells. These findings reveal crucial functions of Foxo1-dependent transcription in control of T cell homeostasis and tolerance.

INTRODUCTION

T lymphocytes are well maintained in the peripheral lymphoid organs under the immune system steady state (Jameson, 2002; Surh and Sprent, 2005). Survival and homeostatic proliferation of T cells is primarily mediated by the cytokine interleukin 7 (IL-7) (Ma et al., 2006; Schluns and Lefrancois, 2003), in part via IL-7 control of expression of target genes including the prosurvival factor Bcl-2 (Akashi et al., 1997; Maraskovsky et al., 1997), and through IL-7 regulation of protein stability of the cyclin-dependent kinase inhibitor p27Kip1 (Barata et al., 2001; Li et al., 2006a). IL-7 exerts its biological effects by binding to the IL-7 receptor complex composed of IL-7 receptor-α chain (IL-7R) and the common γ-chain, resulting in the activation of the Stat5 transcription factor (Jiang et al., 2005). Neutralization of IL-7 results in compromised survival of mature T cells (Schluns et al., 2000; Tan et al., 2001), whereas transgenic expression of IL-7 expands T cells (Kieper et al., 2002). Memory T cells also fail to flourish in IL-7-deficient mice (Kondrack et al., 2003; Li et al., 2003; Schluns et al., 2000; Seddon et al., 2003), emphasizing a prerequisite for IL-7 in control of T cells at various differentiation states. IL-7 is constitutively produced by stromal cells, and the control of IL-7 signaling is largely through the regulation of IL-7R expression in T cells (Mazzucchelli and Durum, 2007). Indeed, IL-7R is not detectable on $CD4^+CD8^+$ immature T cells but is expressed on positively selected mature $CD4^+$ and $CD8^+$ T cells. IL-7R is downregulated upon T cell activation but is re-expressed on memory T cells. Several transcription factors including GABP and Gfi-1 have been shown to regulate IL-7R expression in T cells (Chandele et al., 2008; Park et al., 2004; Xue et al., 2004; Yucel et al., 2003). However, the mechanisms that control IL-7R expression at the various stages of T cell differentiation remain incompletely understood.

The stochastic process by which the T cell receptors are generated creates the inherent problem that some receptors have a high affinity for self-antigens or for innocuous environmental antigens such as those from commensal organisms. Multiple mechanisms have evolved to control T cell-mediated immunopathology, including deletion of self-reactive T cell clones in the thymus and active immune suppression by cytokine TGF-β1 or $CD4^+CD25^+Foxp3^+$ regulatory T cells in the periphery (Li and Flavell, 2008; Mathis and Benoist, 2004; Sakaguchi et al., 2008). In addition to these cell-extrinsic mechanisms, it has been postulated that peripheral T cell tolerance might be regulated by T cell-intrinsic factors including the Forkhead box O (Foxo) family of transcription factors (Yusuf and Fruman, 2003).

Foxo proteins (Foxo1, Foxo3a, Foxo4, and Foxo6) are mammalian homologs of the *Caenorhabditis elegans* transcription factor DAF-16, which have critical functions in control of cellular responses (Burgering, 2008). Foxo activity is downregulated by protein kinase B (PKB)-mediated phosphorylation that triggers nuclear export of Foxo proteins (Huang and Tindall, 2007). In resting T cells, Foxo proteins reside in the nucleus. PKB activation via the stimulation of the T cell receptor, CD28, and cytokine signaling pathways inactivates Foxo proteins, which is associated with the induction of T cell proliferation (Peng, 2008). Indeed, ectopic expression of a PKB-insensitive Foxo1 mutant suppresses T cell proliferation (Fabre et al., 2005; Medema et al., 2000), suggesting that inactivation of Foxo1 is an obligatory step for T cells to enter the cell cycle. Deletion of *Foxo3a* gene in mice results in a mild lymphoproliferative syndrome and the development of inflammatory lesions

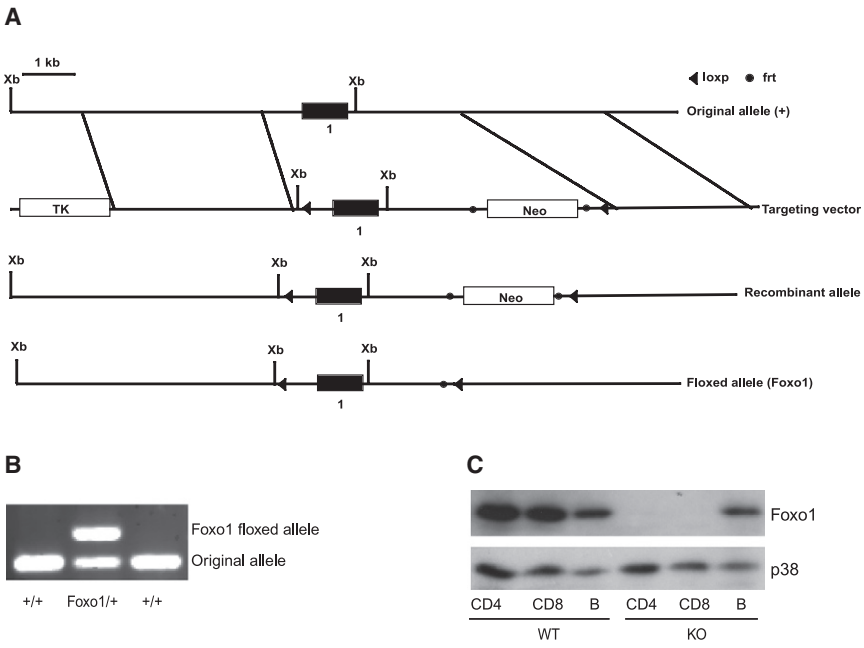

Figure 1. Generation of Mice with T Cell-Specific Deletion of the *Foxo1* Gene

(A) Schematic presentation of original allele (+), targeting vector, the recombinant allele, and the floxed allele (Foxo1). Filled boxes represent the first exon of *Foxo1* gene. The locations of restriction enzyme sites (Xb, Xbal) are indicated. Loxp and Frt sites are shown as arrowheads and filled circles, respectively.

(B) PCR genotyping of the floxed *Foxo1* allele and the original allele.

(C) $CD4^+$ T cells, $CD8^+$ T cells, and B cells were purified from Foxo1/Foxo1 (wild-type, WT) and CD4Cre-Foxo1/Foxo1 (knockout, KO) mice by FACS sorting. The amounts of Foxo1 and p38 were determined by immunoblotting. p38 was used as a loading control.

(Lin et al., 2004), which are associated with spontaneous T cell activation and differentiation. The functions of the other Foxo family proteins in control of T cell responses in vivo remain to be determined.

To investigate the definitive function of Foxo1 in T cells, we generated mice with T cell-specific deletion of the *Foxo1* gene. Thymic T cell differentiation did not appear to be compromised in the absence of Foxo1. However, in the periphery, increased numbers of Foxo1-deficient T cells exhibited an activated phenotype and differentiated into effector T cells, concomitant with the induction of autoantibody. In addition, splenic naive T cell number was reduced in Foxo1-deficient mice. Gene expression profiling of naive T cells revealed novel Foxo1 target genes including *Il7r*. Indeed, expression of IL-7R protein was markedly diminished in Foxo1-deficient naive T cells. Foxo1-deficient naive T cells were refractory to IL-7-induced survival in vitro and exhibited compromised homeostatic proliferation in vivo. Bone marrow chimera experiments revealed that diminished IL-7R expression was a consequence of intrinsic defects of Foxo1-deficient T cells, which was in line with the observation that Foxo1 bound to the evolutionarily conserved regulatory sequences of *Il7r* gene in wild-type T cells. Foxo1-deficient OT-II T cells exhibited a naive T cell phenotype, expressed undetectable amounts of IL-7R, and were depleted from the peripheral lymphoid organs. Re-expression of IL-7R on these cells rescued OT-II T cell number in the periphery. These findings demonstrated a critical role for Foxo1 in control of T cell tolerance and of naive T cell homeostasis through the induction of IL-7R expression.

RESULTS

Generation of Mice with T Cell-Specific Deletion of *Foxo1* Gene

The embryonic lethal phenotype of mice with a germline deletion of *Foxo1* gene prohibited their usage in the study of T cells (Furuyama et al., 2004; Hosaka et al., 2004). To investigate the cell type-specific function of Foxo1 in vivo, we generated mice with a mutated *Foxo1* allele by the insertion of two *loxP* sites flanking its promoter region and the first exon (Figure 1A). The translation start codon of Foxo1 protein resides in exon 1. Deletion of exon 1 was therefore expected to create a null mutation of *Foxo1* gene. *loxP* sites were introduced into the *Foxo1* locus by homologous recombination in mouse embryonic stem (ES) cells. ES cell clones carrying the recombinant Foxo1 locus were used for generating chimeric mice that produced heterozygous mice after germline transmission. These heterozygous mice were bred with a strain of FLP1 recombinase transgenic mice, which led to the excision of the neomycin-resistant gene (*Neo*) flanked by the *frt* sites, and the creation of a floxed *Foxo1* allele (Figure 1B).

Mice with two floxed *Foxo1* alleles developed normally and did not show any sign of disease. These mice, designated as wild-type (WT), were used as the control group in our analysis. To study the function of Foxo1 in T cells, we crossed mice carrying the two floxed *Foxo1* alleles with CD4-Cre transgenic mice, in which Cre is specifically expressed in T cells (Lee et al., 2001). These mice are designated here as Foxo1-deficient mice. Foxo1 protein was not detectable in either $CD4^+$ or $CD8^+$ T cells isolated from Foxo1-deficient mice (Figure 1C), whereas B cells from these mice expressed comparable amounts of Foxo1 to those from WT mice (Figure 1C). These observations reveal efficient and specific ablation of Foxo1 protein in T cells from Foxo1-deficient mice.

T Cell Development in the Absence of T Cell Foxo1

To investigate the consequences of loss of Foxo1 in T cells, we first evaluated thymic T cell development in Foxo1-deficient mice aged between 6 and 8 weeks. The CD4 and CD8 profile of Foxo1-deficient thymocytes was not drastically different from that of WT thymocytes, although a slight increase of TCR-$\beta^{hi}CD4^+$ and TCR-$\beta^{hi}CD8^+$ mature T cells was observed (Figures 2A and 2B). We further examined CD69 and CD62L expression in these T cells and found that upregulation of CD62L was

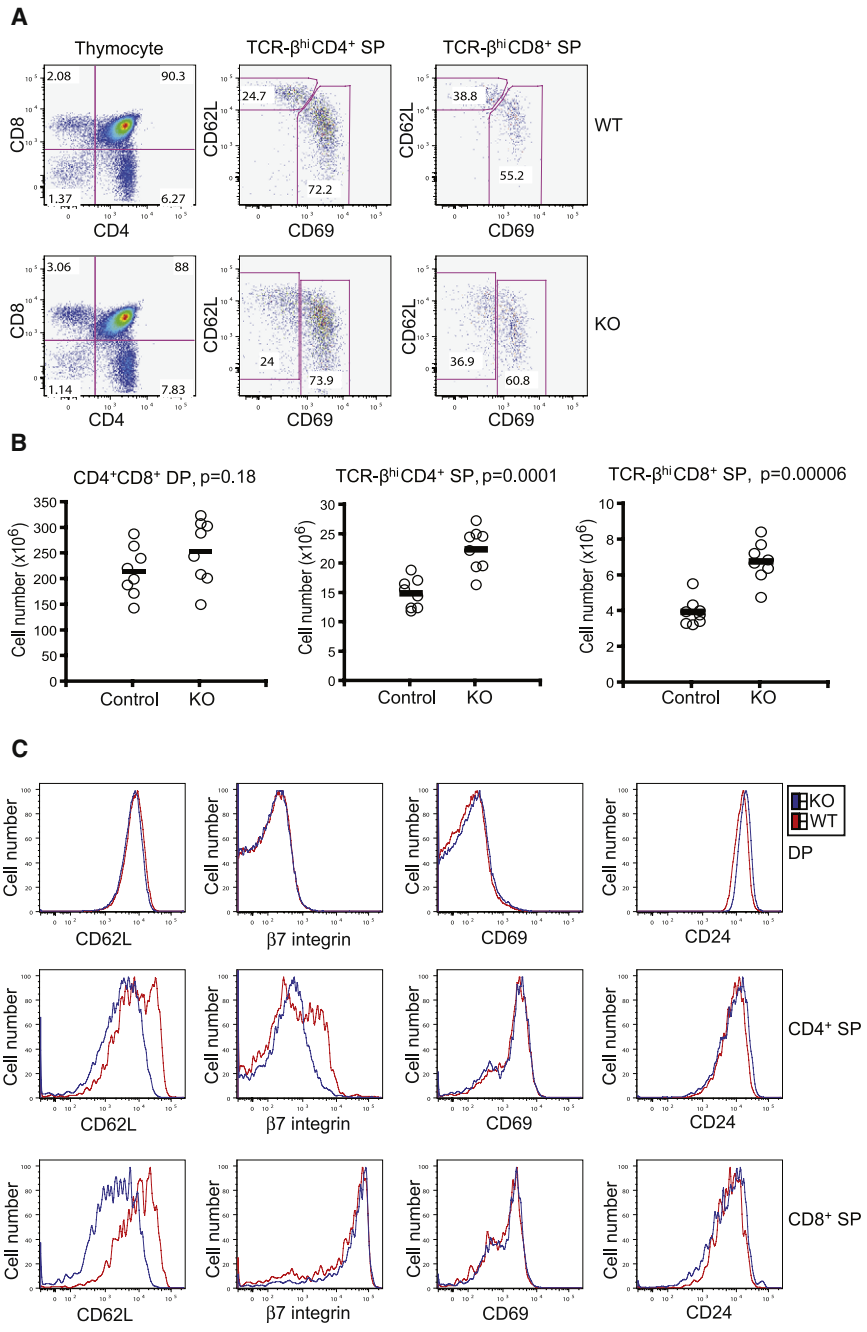

Figure 2. T Cell Development in Foxo1-Deficient Mice

(A) Thymic CD4 and CD8 profile of Foxo1/Foxo1 (wild-type, WT) and CD4Cre-Foxo1/Foxo1 (knockout, KO) mice at 6 weeks old. CD69 and CD62L were measured for TCR-$\beta^{hi}CD4^+$ and TCR-$\beta^{hi}CD8^+$ single-positive (SP) thymocytes by flow cytometry. These are representative results of eight mice per group analyzed from five independent experiments.

(B) Number of thymic $CD4^+CD8^+$ (double-positive, DP), TCR-$\beta^{hi}CD4^+$ SP, and TCR-$\beta^{hi}CD8^+$ SP cells in control (Foxo1/Foxo1) and KO mice (n = 8) at 6–8 weeks old. Each circle represents the cell number from an individual mouse, and the bar represents the mean cell number. The p values between two groups of T cell number are shown.

(C) Expression of CD62L, β7 integrin, CD69, and CD24 in thymocytes from WT and KO mice at 6 weeks old. These are representative results of four mice per group analyzed.

compromised in the $CD69^-$ T cell population from Foxo1-deficient mice (Figures 2A and 2C). These findings are in line with a recent study showing that the expression of a constitutively active form of Foxo1 in human T cells induces CD62L expression, which has been associated with Foxo1 induction of the transcription factor Kruppel-like factor 2 (KLF2) (Fabre et al., 2008). KLF2 is an important regulator of T cell migration (Carlson et al., 2006; Sebzda et al., 2008) and additionally controls the expression of multiple T cell maturation marker proteins including β7 integrin, CD69, and CD24 (Carlson et al., 2006). However, unlike KLF2-deficient T cells, expression of these cell-surface molecules appeared uncompromised in Foxo1-deficient T cells (Figure 2C). Taken together, these observations reveal a specific role for Foxo1 in promoting CD62L expression in mature $CD4^+$ and $CD8^+$ thymocytes in mice.

T Cell Activation and Autoimmunity in T Cell Foxo1-Deficient Mice

A previous study of Foxo3a-deficient mice showed that Foxo3a is essential for the inhibition of T cell activation and effector T cell differentiation (Lin et al., 2004). To investigate the function of Foxo1 in control of peripheral T cells, we first examined the expression of T cell activation markers CD44, CD62L, and CD69 in $CD4^+$ and $CD8^+$ T cells isolated from the spleens of WT and Foxo1-deficient mice. Compared to WT T cells, a higher percentage of Foxo1-deficient T cells exhibited an activated $CD44^{hi}CD62L^{lo}$ or $CD69^+$ phenotype (Figures 3A and 3C). Notably, similar to Foxo1-deficient thymic mature T cells, the $CD44^{lo}$ naive $CD4^+$ and $CD8^+$ T cells from Foxo1-deficient mice expressed lower amounts of CD62L than did control T cells from WT mice (Figures 3A and 3C). Increased T cell activation and decreased CD62L expression in naive T cells was also observed in the lymph nodes of Foxo1-deficient mice (Figures S1 and S2 available online). In addition, Foxo1-deficient mice developed lymphadenopathy associated with the expansion of $CD4^+$ T cells that expressed high amounts of the proliferating cell marker Ki-67 antigen (Figures S1 and S2). The cellularity of Foxo1-deficient spleens was not markedly different from that of WT spleens (data not shown). However, spleens from Foxo1-deficient mice had increased number of $CD4^+$ and

CD8$^+$ T cells with the activated CD44^{hi}CD62L^{lo} phenotype, whereas the number of CD44^{lo}CD62L^{hi} naive CD4$^+$ and CD8$^+$ T cells was decreased (Figure 3B). In addition to conventional CD4$^+$ and CD8$^+$ T cells, CD4$^+$CD8$^+$ immature T cells also give rise to a CD4$^+$CD25$^+$ regulatory T (Treg) cell lineage that expresses the transcription factor Foxp3 (Sakaguchi et al., 2008). CD4$^+$CD25$^+$Foxp3$^+$ Treg cells are essential regulators of peripheral T cell tolerance. To determine whether enhanced T cell activation in Foxo1-deficient mice was caused by the depletion of Treg cells, we examined these cells in the thymus and in the periphery. A comparable percentage of Foxp3-positive Treg cells was found in both WT and Foxo1-deficient mice in all lymphoid organs examined (Figure 3D; Figure S1). To investigate whether Foxo1 deficiency affected Treg cell function, we crossed Foxo1-deficient mice with Foxp3-RFP reporter mice (Wan and Flavell, 2005) and isolated Treg cells on the basis of RFP expression. Foxo1-deficient Treg cells inhibited naive T cell proliferation as potently as did WT Treg cells in an in vitro assay (Figure S3). Taken together, these findings suggest a cell-intrinsic function for Foxo1 in the maintenance of naive phenotype T cells and in the prevention of T cell activation.

Foxo1-deficient CD4$^+$ and CD8$^+$ T cells also expressed higher amounts of surface CD122 (Figure 3C), the shared receptor for IL-2 and IL-15 (Ma et al., 2006). We and others have shown that CD122 expression is controlled by transcription factors T-bet and eomesodermin that also regulate Th1 and cytotoxic T lymphocyte (CTL) differentiation (Intlekofer et al., 2005; Li et al., 2006b). To determine effector T cell differentiation in Foxo1-deficient mice, we stimulated T cells from spleens with PMA and ionomycin for 4 hr and performed intracellular cytokine staining. Compared to T cells from WT mice, which had only a few CD4$^+$ and CD8$^+$ T cells capable of producing effector cytokines IFN-γ, IL-4, IL-10, and IL-17, a higher percentage of CD4$^+$ and CD8$^+$ T cells from Foxo1-deficient mice produced these cytokines (Figure 3E). A similar increase in the number of cytokine-producing T cells was also observed in the lymph nodes of Foxo1-deficient mice (Figures S1 and S2). To determine whether the increased cytokine production of Foxo1-deficient T cells was a consequence of enhanced T cell activation, we determined the frequency of cytokine-producing T cells among the activated CD44^{hi} T cells from WT and Foxo1-deficient mice. A higher percentage of Foxo1-deficient CD4$^+$CD44^{hi} T cells produced IFN-γ or IL-17 than did WT CD4$^+$CD44^{hi} T cells, whereas IL-4- or IL-10-producing CD4$^+$ T cells or IFN-γ-producing CD8$^+$ T cells were comparable among the activated WT and Foxo1-deficient T cells (Figure S4). These observations suggest that in addition to Foxo1 control of T cell activation, Foxo1 plays a major role in inhibiting Th1 and Th17 cell differentiation. To investigate whether this enhanced T cell differentiation would trigger immunopathology, we aged a cohort of Foxo1-deficient mice for 5–6 months. Histopathological examination did not reveal drastic inflammation in any of the major organs (data not shown). We also measured the amounts of autoreactive antibodies in these mice. Increased titers of both nuclear and dsDNA antibodies were detected in the sera of Foxo1-deficient mice (Figure 3F). These observations demonstrate that T cell Foxo1 is essential for the inhibition of effector T cell differentiation and for the maintenance of B cell tolerance to self-antigens.

Foxo1-Dependent Transcriptional Program in Naive T Cells

We wished to identify Foxo1 target genes that are involved in the control of T cell homeostasis and tolerance. To this end, we purified naive phenotype (CD44^{lo}CD62L^{hi}CD25$^-$) CD4$^+$ and CD8$^+$ T cells from WT and Foxo1-deficient mice by FACS sorting. RNA was prepared from these cells and was analyzed by global gene expression profiling with the Affymetrix oligonucleotide arrays. Comparing WT and Foxo1-deficient CD4$^+$ T cells, 396 entries showed equal or greater than 2-fold change, whereas 497 entries differed in CD8$^+$ T cells (Figure 4A, for a complete list see Tables S1 and S2). Among the differentially expressed entries, 187 were shared between CD4$^+$ and CD8$^+$ T cells (Figures 4B and 4C, for a complete list see Table S3). We initially focused our analysis on these coregulated genes, which encode (among others) cell-surface molecules, nuclear factors, and proteins involved in the signal transduction and metabolism (Figure 4C). Notably, the expression of genes encoding positive regulators of T cell activation and differentiation such as *Tnfrsf9, Gadd45 g*, and *Rora* was increased in Foxo1-deficient T cells (Figure 4C; Lu et al., 2001; Vinay and Kwon, 1998; Yang et al., 2008). In addition, Foxo1 controls the expression of genes involved in cell adhesion, cell migration, and cellular stress responses (Figure 4C). These findings reveal diverse Foxo1 target genes in T cells that may collectively control T cell homeostasis and tolerance.

Foxo1 Regulation of IL-7R Expression and IL-7 Signaling in T Cells

We focused on one of the Foxo1 target genes, *Il7r,* which was downregulated in both CD4$^+$ and CD8$^+$ Foxo1-deficient T cells (Figure 4C). In line with the gene expression results, Foxo1-deficient CD4$^+$ and CD8$^+$ CD44^{lo}CD62L^{hi} naive T cells expressed low to undetectable amounts of IL-7R (CD127) protein (Figure 5A, left). IL-7R expression is induced in the thymocytes that undergo positive selection (Mazzucchelli and Durum, 2007). In contrast to the upregulation of IL-7R in WT thymocytes, Foxo1-deficient CD4$^+$ and CD8$^+$ T cells expressed increasingly lower amounts of IL-7R when they matured from CD69$^+$CD62L^{lo} to CD69$^-$CD62L^{hi} T cells (Figure 5B). IL-7R expression was also greatly diminished in the activated CD44^{hi} Foxo1-deficient T cells (Figure 5A, right). These observations reveal a critical role for Foxo1 in control of IL-7R expression at multiple stages of T cell differentiation.

The IL-7-IL-7R pathway is a pivotal regulator of T cell homeostasis, which is in part mediated by its induction of the prosurvival *Bcl2* gene expression (Akashi et al., 1997; Maraskovsky et al., 1997). Consistent with the reduced IL-7R expression, Foxo1-deficient CD4$^+$ and CD8$^+$ T cells expressed lower amounts of Bcl-2 protein than did WT T cells (Figure 5C). IL-7 engagement of IL-7R activates JAK3 and JAK1 kinases that phosphorylate the Stat5 transcription factor (Jiang et al., 2005). Unlike WT naive T cells, IL-7 stimulation of Foxo1-deficient T cells failed to induce Stat5 phosphorylation (Figure 5D). IL-7 is a potent regulator of naive T cell survival. Stimulation of WT CD4$^+$ or CD8$^+$ naive T cells with IL-7 triggered dose-dependent inhibition of cell apoptosis assessed with Annexin V staining (Figure 5E). However, both CD4$^+$ and CD8$^+$ Foxo1-deficient naive T cells were refractory to IL-7-induced survival in vitro (Figure 5E). In vivo, IL-7 regulates the survival and homeostatic proliferation of naive T cells (Ma et al., 2006). To investigate the

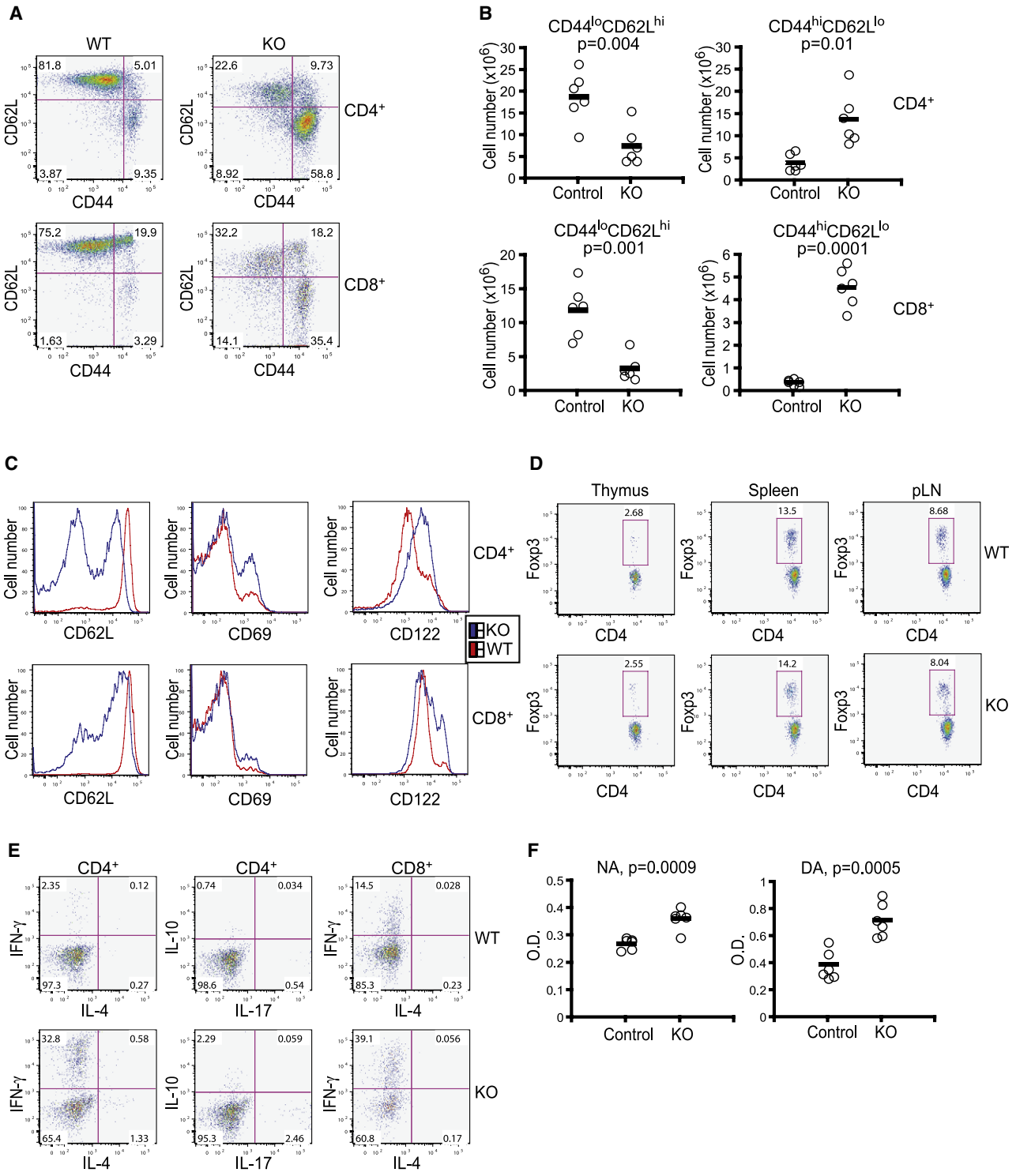

Figure 3. T Cell Activation and Differentiation in Foxo1-Deficient Mice

(A) Expression of CD44 and CD62L in splenic $CD4^+$ and $CD8^+$ T cells from Foxo1/Foxo1 (wild-type, WT) and CD4Cre-Foxo1/Foxo1 (knockout, KO) mice at 6 weeks old. These are representative results of six independent experiments.

(B) The number of splenic $CD4^+$ and $CD8^+$ naive ($CD44^{lo}CD62L^{hi}$) and activated ($CD44^{hi}CD62L^{lo}$) T cells from control (Foxo1/Foxo1) and KO mice (n = 6) at 6–8 weeks old. The p values between two groups of T cell number are shown.

(C) Expression of CD62L, CD69, and CD122 in splenic $CD4^+$ and $CD8^+$ T cells from WT and KO mice at 6 weeks old. These are representative results of six mice per group analyzed.

proliferation potential of Foxo1-deficient T cells, we performed a transfer experiment. We purified wild-type naive $CD4^+$ or $CD8^+$ T cells from C57BL/6 mice that expressed the congenic marker CD45.1. These T cells were mixed with Foxo1-deficient naive T cells expressing the congenic marker CD45.2 at approximately 1:1 ratio (Figure 5F, top), labeled with CFSE, and transferred to *Rag1*$^{-/-}$ recipients. The usage of the CD45 marker enabled us to differentiate WT and Foxo1-deficient T cells. After 7 days, T cells were recovered from the spleens and lymph nodes of the recipient mice and assessed for cell proliferation by CFSE dilution. We found that the recovery of Foxo1-deficient T cells was about 10%–20% of the WT T cells, which was associated with the compromised homeostatic proliferation of Foxo1-deficient T cells (Figure 5F). These observations further corroborated that the IL-7R expression defect of Foxo1-deficient T cells caused compromised IL-7 signaling and IL-7-induced T cell survival and proliferation.

A Cell-Intrinsic Role for Foxo1 in Control of IL-7R Expression in T Cells

IL-7R expression is subject to regulation by multiple environmental cues such as the presence of other prosurvival cytokines including IL-2, IL-4, IL-6, and IL-15 (Park et al., 2004). This has been postulated as a mechanism to promote survival of the maximum possible number of T cells for the limited amount of IL-7 available (Mazzucchelli and Durum, 2007). Because a large fraction of Foxo1-deficient T cells were activated and produced effector cytokines (Figure 3), it was possible that the downregulation of IL-7R expression in Foxo1-deficient T cells was a consequence of the heightened cytokine stimulation. To study whether Foxo1 control of IL-7R expression was via cell-intrinsic or cell-extrinsic pathways, we generated mixed bone marrow chimeric mice. T cell-depleted bone marrow cells from $CD45.2^+$ Foxo1-deficient mice and $CD45.1^+$ WT mice were transferred either separately or in combination into sublethally irradiated *Rag1*$^{-/-}$ recipients. All chimeric mice reconstituted with Foxo1-deficient bone marrow cells developed severe wasting disease 8 weeks after the transfer. Upon histological examination, we found heavy mononuclear cell infiltration in the mucosal lamina propria and the subglandular area of the colons of these mice (Figure 6A). In contrast, mice reconstituted with WT bone marrow cells did not develop colitis (Figure 6A). A higher proportion of splenic $CD4^+$ and $CD8^+$ T cells from the Foxo1-deficient chimera exhibited an activated phenotype than did T cells from the WT chimera (Figure 6B) and differentiated to cytokine-producing effector T cells (data not shown). To determine whether Foxo1 deficiency affected Treg cell homeostasis under these conditions, we assessed Treg cell frequencies in these mice. Approximately 30% of $CD4^+$ T cells from the WT chimera were Treg cells, compared to about 7% of Foxo1-deficient $CD4^+$ T cells (Figure 6C). These observations demonstrate an important role for Foxo1 in control of T cell tolerance, T cell activation, and Treg cell homeostasis in the bone marrow chimeric mice.

Mixed bone marrow chimeric mice, however, did not develop colitis (Figure 6A). To examine the reconstitution efficiency of WT and Foxo1-deficient bone marrow, we examined the distribution of $CD45.1^+$ WT T cells and $CD45.2^+$ Foxo1-deficient T cells in these mice. In the thymus of one of the mixed chimeras, WT and Foxo1-deficient T precursor cells produced comparable number of TCR-β^{hi} mature T cells (Figure 6D, top). However, in the spleen and lymph nodes of this mouse, the number of $CD45.2^+$ Foxo1-deficient T cells was greatly diminished compared to that of $CD45.1^+$ WT T cells (Figure 6D, middle and bottom). Normalized to the reconstitution efficiency of the thymus, the number of Foxo1-deficient $CD4^+$ and $CD8^+$ T cells in the periphery were 6%–30% that of WT T cells (Figure 6E). Similar to T cells from WT or Foxo1-deficient chimeras, Foxo1-deficient population of $CD4^+$ and $CD8^+$ T cells from the mixed chimeras expressed lower amounts of IL-7R than did WT population in the same mouse (Figure 6F). These observations reveal a T cell-intrinsic role for Foxo1 in promoting IL7-R expression, which was associated with the out competition of Foxo1-deficient T cells by WT T cells in the periphery. To determine whether Foxo1 directly controlled *Il7r* gene transcription, we searched for evolutionarily conserved Foxo1-binding sites in the mouse *Il7r* promoter. With rVista program, we found three putative Foxo1-binding sites within the 10 kb *Il7r* promoter region that were conserved between mouse and human (Figure S5). To investigate whether Foxo1 directly bound to these DNA elements, we performed chromatin immunoprecipitation of WT T cells with Foxo1 or control antibodies. Genomic fragments containing the proximal *Il7r* promoter, the 3.7 kb, but not the 9.1 kb, DNA regions upstream of the translation start site were selectively enriched with the Foxo1 antibody (Figures 6G and 6H). These findings support *Il7r* as a direct Foxo1 target gene in T cells.

A Critical Function of IL-7R in Foxo1 Control of Naive OT-II T Cell Homeostasis

Foxo1 deficiency in T cells resulted in the activation and differentiation of a fraction of the peripheral T cells (Figure 3). We wished to determine how the antigen specificity of T cell receptor (TCR) would influence T cell responses in the absence of Foxo1. To this end, we crossed Foxo1-deficient mice with OT-II ($CD4^+$ TCR specific for an OVA peptide) transgenic mice onto *Rag1*$^{-/-}$ background, in order to exclude the T cell repertoire as a variable. Similar to T cells on the polyclonal background (Figure 2), deficiency of Foxo1 did not compromise OT-II T cell-positive selection (Figure S6). In addition, mature thymic OT-II T cells expressed lower amounts of CD62L and IL-7R (Figure S6), which was also in line with the polyclonal T cells (Figures 2 and 5). However, unlike T cells on the polyclonal background, splenic Foxo1-deficient OT-II T cells maintained a naive $CD44^{lo}$ phenotype (Figure 7A). Compared to WT OT-II mice, the number of

(D) The percentage of thymic, splenic, and peripheral lymph node (pLN) $CD4^+Foxp3^+$ regulatory T (Treg) cells in WT and KO mice at 6 weeks old. These are representative results of six mice per group analyzed.
(E) $CD4^+$ and $CD8^+$ T cells isolated from the spleens of WT and KO mice were stimulated with PMA and ionomycin for 4 hr and analyzed for the expression of IFN-γ, IL-4, IL-10, and IL-17 by intracellular cytokine staining. These are representative results of two independent experiments.
(F) Titers of nuclear antibody (NA) and dsDNA antibody (DA) in the sera of control and KO mice aged between 5 and 6 months (n = 6). The p values between the two groups of antibody titers are shown.

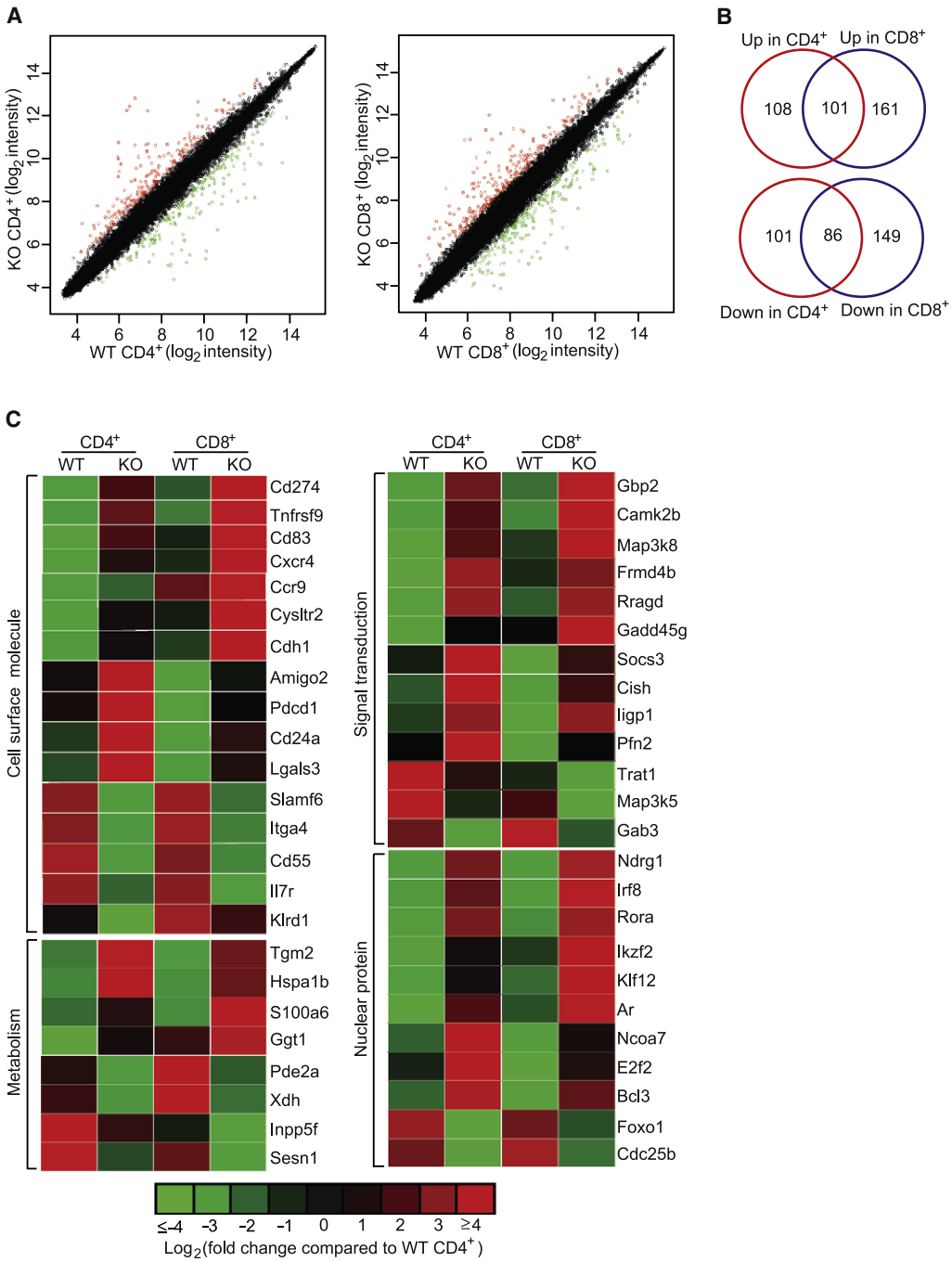

Figure 4. Differential Gene Expression between Wild-type and Foxo1-Deficient Naive T Cells

(A) Comparison of gene expression values between Foxo1/Foxo1 (wild-type, WT) and CD4Cre-Foxo1/Foxo1 (knockout, KO) naive $CD4^+$ and $CD8^+$ T cells. Data analyses were done with R Console. The genes with 2-fold or more change of expression were marked as red or green circles.

(B) Overlapping of Foxo1-regulated genes in naive $CD4^+$ and $CD8^+$ T cells. Genes with expression values equal or more than 2-fold different between WT and KO T cells are considered significant. The numbers of shared or uniquely regulated genes between $CD4^+$ and $CD8^+$ T cells are indicated.

(C) A sublist of coregulated genes between $CD4^+$ and $CD8^+$ T cells. The genes were analyzed for gene ontology and are grouped as cell-surface molecules, signaling proteins, nuclear factors, and molecules involved in cell metabolism.

mature OT-II T cells was greatly reduced in the spleens and lymph nodes of Foxo1-deficient OT-II mice by 80%–90% (Figure 7B). These observations reveal that Foxo1 is essential for the maintenance of naive OT-II T cells in vivo.

As expected, Foxo1-deficient OT-II T cells failed to express IL-7R (Figure 7C). To determine the functional consequences of diminished IL-7R expression in Foxo1-deficient T cells, we crossed Foxo1-deficient mice with IL-7R transgenic (IL-7RTg)

mice (Park et al., 2004). Restoration of IL-7R expression did not correct the T cell activation phenotype or significantly affect the number of Foxo1-deficient T cells on the polyclonal background (Figure S7 and data not shown). However, the restored IL-7R expression rescued peripheral T cell number in Foxo1-deficient OT-II IL-7RTg mice (Figures 7D and 7E), which was associated with the recovery of Bcl-2 protein expression in Foxo1-deficient OT-II T cells (Figure 7F). These findings establish a central role for IL-7R in Foxo1 control of naive T cell homeostasis.

DISCUSSION

Because of the embryonic lethal phenotype of Foxo1-deficient mice, the function of Foxo1 in T cells has not been studied in vivo. We have developed a mouse strain that enabled cell type-specific deletion of *Foxo1* gene via the Cre-loxP system. In this report, we used CD4-Cre transgenic mice to delete *Foxo1* gene in T cells and explored its role in thymic T cell development and peripheral T cell activity. We found that Foxo1 was not essential for the positive selection of $CD4^+$ and $CD8^+$ T cells, but was required for the expression of IL-7R and CD62L in mature thymocytes. Foxo1 deficiency also led to the compromised IL-7R and CD62L expression in naive T cells in the peripheral lymphoid organs. Diminished expression of IL-7R was associated with failed IL-7 signaling in Foxo1-deficient T cells, which resulted in the compromised IL-7-induced T cell survival in vitro and reduced IL-7-dependent homeostatic proliferation in vivo. During revision of the current manuscript, Kerdiles et al. (2009) published a report on the use of a different strain of T cell-specific Foxo1-deficient mice to reveal Foxo1 regulation of IL-7R and CD62L expression similar to findings in our study. However, the functional significance of diminished IL-7R expression in Foxo1-deficient T cells was not addressed in that study. By using a strain of IL-7R transgenic mouse, we showed that reduced IL-7R expression was responsible for the homeostasis defects of naive Foxo1-deficient OT-II T cells. In addition, Foxo1 deficiency caused spontaneous T cell activation, effector T cell differentiation, and the production of autoantibodies in mice. In a bone marrow transfer model, lack of Foxo1 expression in T cells resulted in colitis. These observations reveal previously undefined potent and pleiotropic roles for Foxo1 in control of T cell homeostasis and tolerance in vivo.

A major finding of the present study was that Foxo1 controlled naive T cell homeostasis via its regulation of IL-7R expression. As a transcription factor, Foxo1 can bind to regulatory DNA sequences on target genes (Obsil and Obsilova, 2008). Indeed, we identified consensus Foxo1-binding sites in the promoter region of *Il7r* gene. We further found direct Foxo1 association with the proximal *Il7r* promoter and an evolutionarily conserved noncoding region 3.7 kb upstream of the translation start site. Future studies will be needed to test the importance of these Foxo1 binding sites in control of IL-7R expression in T cells. In addition, it has been reported that Foxo1 can regulate gene expression independent of its DNA-binding domain (Ramaswamy et al., 2002). In this case, Foxo1 may interact with other nuclear factors involved in the control of IL-7R expression. Previous studies have revealed that IL-7R transcription in T cells is positively regulated through proximal promoter region that contains binding motifs for the transcription factor GABP (Xue et al., 2004). IL-7R transcription is also subjected to repression by the transcription repressor Gfi-1 (Park et al., 2004), which binds to an intronic region of *Il7r* gene. How Foxo1 interacts with these transcription factors in control of IL-7R transcription will be an interesting area for future exploration.

The expression of IL-7R is dynamically regulated at multiple stages of T cell differentiation (Mazzucchelli and Durum, 2007). When naive T cells encounter antigen during infection, they undergo expansion and differentiation. This is associated with the downregulation of IL-7R expression on most effector T cells (Huster et al., 2004; Kaech et al., 2003). Stimulation of T cells via the TCR, costimulatory receptor, and cytokine signaling pathways also inactivates Foxo1 via PKB-induced phosphorylation (Peng, 2008). It remains to be determined whether the downregulation of IL-7R expression on effector T cells is a consequence of Foxo1 inactivation. It has been shown that a small subset of the effector $CD8^+$ T cells express high amounts of IL-7R and differentiate into long-lived memory $CD8^+$ T cells (Huster et al., 2004; Kaech et al., 2003). The function of Foxo1 in control of IL-7R expression in memory T cells warrants further investigation.

In addition to the control of naive OT-II T cell homeostasis, Foxo1 was required for the inhibition of T cell activation and differentiation on T cell polyclonal background. It has been proposed that T cell activation and development of autoimmune diseases can be caused by T cell lymphopenia (King et al., 2004; Marleau and Sarvetnick, 2005), which is associated with IL-7-driven homeostatic T cell proliferation (Calzascia et al., 2008). Foxo1-deficient naive T cells were depleted and expressed markedly lower amounts of IL-7R than did Foxo1-deficient T cells with the activated phenotype, raising the possibility that T cell activation was a consequence of enhanced IL-7 stimulation. Overexpression of IL-7R via an IL-7R transgene in Foxo1-deficient T cells largely nullified IL-7R expression difference between naive and activated T cells, but did not correct the T cell activation phenotype. These observations suggest that T cell activation in the absence of Foxo1 was not caused by defective IL-7R expression. Treg cell number was not reduced in unmanipulated Foxo1-deficient mice, which is consistent with a dispensable role for the IL-7R signaling pathway in control of Treg cell homeostasis (Bayer et al., 2008; Mazzucchelli et al., 2008; Vang et al., 2008). These findings imply that Foxo1 functions as a T cell-intrinsic regulator of tolerance. The mechanisms by which Foxo1 regulates T cell activation remain to be determined. Gene expression profiling experiments revealed hundreds of putative Foxo1 target genes in naive T cells. However, it is still an open question whether Foxo1 controls another "master" regulator of T cell tolerance, or alternatively Foxo1 regulates multiple signaling pathways that collectively ensure naive T cell quiescence.

Reconstitution of sublethally irradiated $Rag1^{-/-}$ mice with Foxo1-deficient bone marrow cells resulted in severe colitis that was not observed in unmanipulated Foxo1-deficient mice aged for 5–6 months. Whole-body irradiation induces tissue damage and triggers the release of microbes and microbial products that cause systemic inflammation (Paulos et al., 2007). It remains to be determined whether the heightened

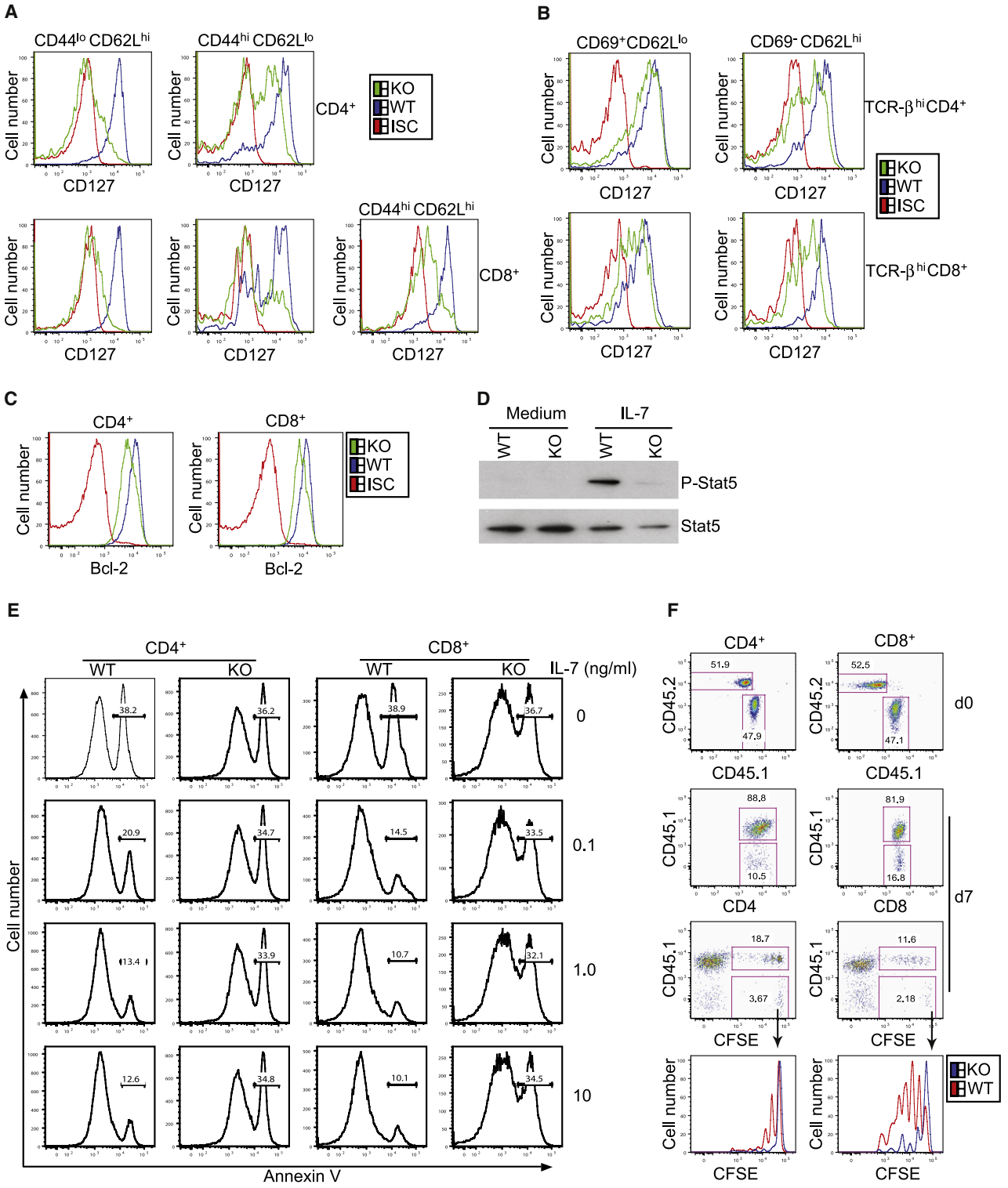

Figure 5. Defective IL-7R Expression and IL-7 Signaling in Foxo1-Deficient T Cells

(A) Expression of CD127 in splenic CD4$^+$ and CD8$^+$ T cells from Foxo1/Foxo1 (wild-type, WT) and CD4Cre-Foxo1/Foxo1 (knockout, KO) mice at 6 weeks old. CD44^{lo}CD62L^{hi}, CD44^{hi}CD62L^{lo}, or CD44^{hi}CD62L^{hi} T cells represent naive, effector, and central memory subsets, respectively. ISC stands for the isotype control antibody. These are representative results of five independent experiments.

(B) Expression of CD127 (IL-7R) in thymic TCR-β^{hi}CD4$^+$ and TCR-β^{hi}CD8$^+$ T cells from WT and KO mice at 6 weeks old. CD69$^+$CD62L^{lo} or CD69$^-$CD62L^{hi} T cells represent immature and mature populations, respectively. These are representative results of five independent experiments.

inflammatory response associated with irradiation contributes to the development of colitis in Foxo1-deficient chimeras. In Foxo1-deficient chimeric mice, the number of $CD4^+Foxp3^+$ cells was diminished compared to that in the WT chimeras. Reduced percentage of Foxo1-deficient Treg cells was also observed in the mixed chimeric mice that had received both wild-type and Foxo1-deficient bone marrows. These findings reveal a cell-intrinsic role for Foxo1 in control of Treg cell homeostasis in irradiated mice. Active immune suppression by Treg cells is essential for T cell tolerance (Sakaguchi et al., 2008). How Foxo1 cross-talks with Treg cells in control of T cell responses will be an interesting area for future study. In contrast to T cells from Foxo1-deficient chimeras, Foxo1-deficient T cell populations from the mixed chimeric mice exhibited a naive T cell phenotype. Replenishment of WT Treg cell in the mixed chimeras might suppress the activation of Foxo1-deficient T cells. Foxo1-deficient T cells expressed low amounts of IL-7R and were not competitive to WT T cells in the periphery. Therefore, it is also possible that Foxo1-deficient T cells were rapidly depleted upon release from the thymus, before they could be activated by peripheral antigens. Because T cell activation in unmanipulated Foxo1-deficient mice was not associated with observable Treg cell defects, Foxo1 likely played an autonomous role in control of T cell activation.

The nature of the antigens that drive the expansion and differentiation of effector T cells in Foxo1-deficient mice remains to be fully characterized. Interestingly, Foxo1-deficient OT-II T cells on the *Rag1*$^{-/-}$ background were not activated. Because OT-II T cells are specific for the foreign ovalbumin antigen, these results imply that cognate antigen stimulation is needed for the activation of Foxo1-deficient T cells. Increased production of nuclear and dsDNA antibodies in Foxo1-deficient mice further suggested that self-antigens might be involved in the activation of T cells. Although we did not observe spontaneous colitis in Foxo1-deficient mice aged up to 6 months, T cells isolated from the gut-draining mesenteric lymph node exhibited more pronounced T cell activation than T cells from the other peripheral lymph nodes. Importantly, transfer of bone marrow cells isolated from T cell-specific Foxo1-deficient mice into irradiated *Rag1*$^{-/-}$ mice led to the development of colitis in recipient mice. These observations imply that Foxo1 is also critical to prevent the activation of T cells reactive to commensal bacterium antigens.

In conclusion, in this report, we have uncovered critical functions for Foxo1 in regulation of T cell homeostasis and tolerance. IL-7R was identified as a Foxo1 target gene involved in Foxo1 maintenance of naive T cells. These findings will advance our knowledge on the function of Foxo family proteins in the immune system and might, in the long term, be exploited for finding cures for autoimmune diseases and cancer.

EXPERIMENTAL PROCEDURES

Mice

Mouse genomic DNA of the *Foxo1* gene was isolated from a 129SV BAC library (Genome Systems). The targeting vector was constructed by cloning three genomic fragments into the plasmid of pEasy-FLIRT. Linearized targeting vector was transfected into ES cells (TC1). Homologous recombinants were identified by Southern blot analysis and were implanted into foster mothers. Chimeric mice were bred to C57BL/6 mice, and the F1 generation was screened for germline transmission. The *Neo* gene was removed by breeding F1 mice with a strain of actin promoter-driven Flipase transgenic mice (JAX). Mice carrying the floxed allele of Foxo1 were backcrossed to C57BL/6 for five to six generations. CD4-Cre transgenic mice, OT-II TCR transgenic (OT-II) mice, and Foxp3-RFP knockin mice were described previously (Li et al., 2006b; Wan and Flavell, 2005). *IL-7R* transgenic (IL-7RTg) mice (Park et al., 2004) were kindly provided to us by A. Singer (National Cancer Institute). All mice were maintained under specific pathogen-free conditions. All animal experimentation was conducted in accordance with institutional guidelines.

Histopathology

Tissues from sacrificed animals were fixed in Safefix II (Protocol) and embedded in paraffin. 5 μm sections were stained with hematoxylin and eosin.

Immunoblotting

For the analysis of Foxo1 protein expression, FACS-sorted $CD4^+$ T, $CD8^+$ T, and B cells were extracted with 1 × SDS sample buffer. To analyze IL-7-stimulated Stat5 phosphorylation, FACS-sorted naive ($CD44^{lo}CD62L^{hi}CD25^-$) $CD4^+$ and $CD8^+$ T cells from WT and Foxo1-deficient mice were left untreated or treated with 10 ng/ml IL-7 for 20 min and were lyzed with 1 × SDS sample buffer. Protein extracts were separated on 8% SDS-PAGE gels and transferred to PVDF membrane (Millipore). The membranes were probed with antibodies against Foxo1, p38, Stat5, and phosphorylated Stat5 (Cell Signaling).

Chromatin Immunoprecipitation

The chromatin immunoprecipitation analysis was performed as described previously (Li et al., 2006b). In brief, $CD4^+$ T cells were fixed for 10 min at room temperature with 10% formaldehyde. After incubation, glycine was added to a final concentration of 0.125 M to "quench" the formaldehyde. Cells were pelleted, washed once with ice-cold PBS, and then lysed. The lysates were pelleted, resuspended, and sonicated to reduce DNA length to between 500 and 1000 base pairs. The chromatin was precleared with protein A agarose beads for 1 hr and then incubated with 5 μg of Foxo1 antibody (ab39670, Abcam) or control rabbit Ig overnight. The immune complexes were precipitated with protein A agarose beads, washed, and eluted in 100 μl of TE with 0.5% SDS and 200 μg/ml proteinase K. Precipitated DNA was further purified with phenol/chloroform extranction and ethanol precipitation and was analyzed by quantitative PCR (qPCR). The primers used in the analysis of binding included: −9201 to −8993: 5′-GCC CAC TTT CTC CCT ACC TT-3′ and 5′-TCC AGC TAC TTC ACC GAA GC-3′; −3856 to −3545: 5′-TCT TTA AGC TTC CCG CAC TC-3′ and 5′-ACC TCA TCA GCC TTT CAT GG-3′; −385 to −88: 5′-AAG TGT GGA TTT TGG CCT TG-3′ and 5′-GAG AGA GGG AGA CCC AAA CC-3′.

Flow Cytometry

Cells from spleens, lymph nodes, or thymus were depleted of erythrocytes by hypotonic lysis. Cells were incubated with specific antibodies for 15 min on ice in the presence of 2.4G2 mAb to block FcγR binding. Samples were analyzed

(C) Expression of Bcl-2 in splenic $CD4^+$ and $CD8^+$ naive T cells from WT and KO mice at 8 weeks old. Intracellular staining was performed, and Bcl-2 expression was determined by flow cytometry.
(D) Naive $CD4^+$ T cells from WT and KO mice were left untreated (medium) or treated with 10 ng/ml IL-7 for 20 min. The amounts of phosphorylated Stat5 and total Stat5 protein were determined by immunoblotting.
(E) Naive $CD4^+$ and $CD8^+$ T cells from WT and KO mice were cultured in the absence or presence of increasing doses of IL-7 for 24 hr. Cell apoptosis was examined by Annexin-V staining.
(F) Naive $CD4^+$ and $CD8^+$ T cells isolated from $CD45.2^+$ KO and $CD45.1^+$ congenic WT mice were labeled with CFSE and cotransferred into Rag1-deficient mice. The percentage of WT and KO T cells before (d0) and after (d7) transfer was shown. CFSE dilution indicates cell proliferation. The histograms showed homeostatic proliferation of the transferred T cells. These are representative results of three independent experiments.

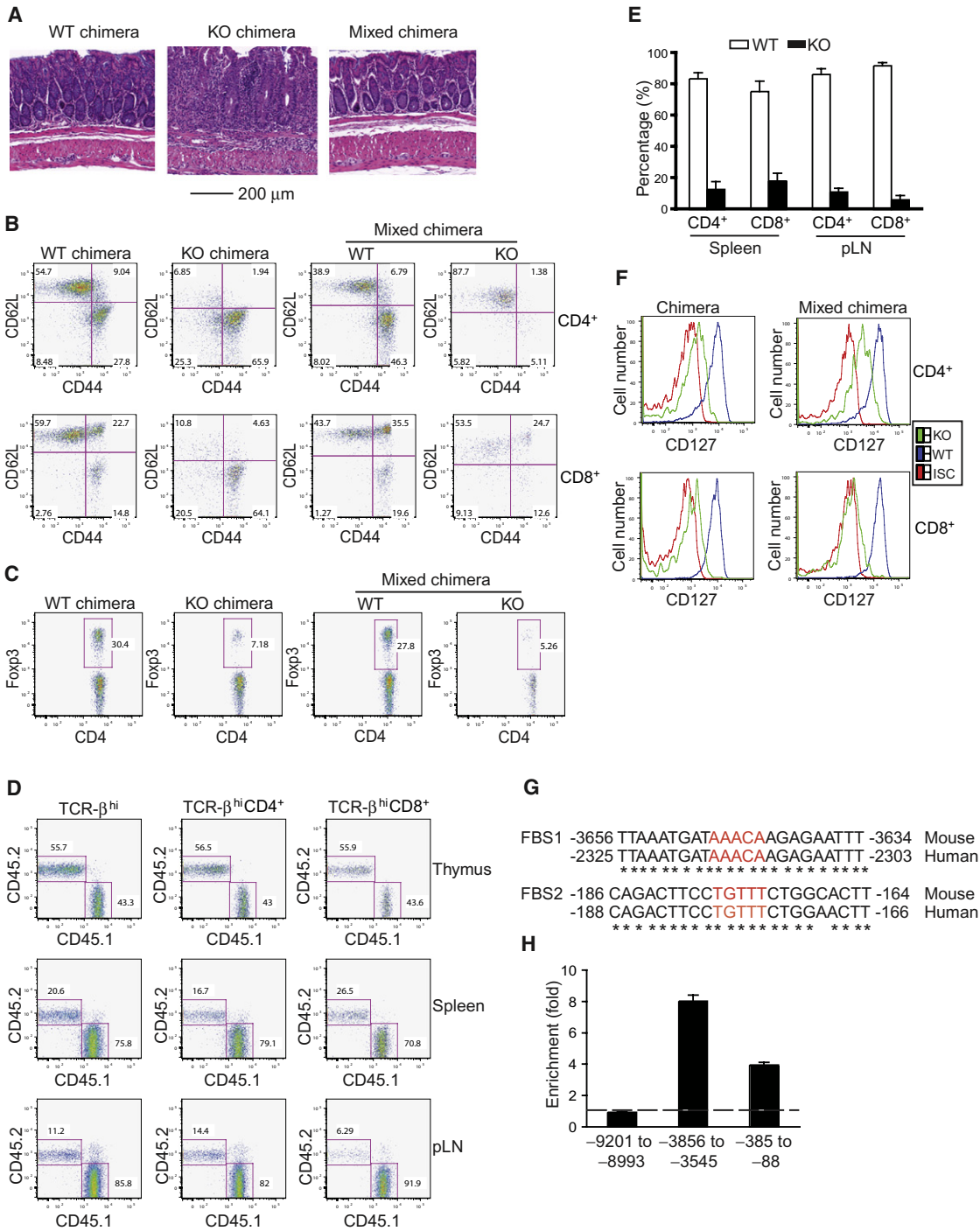

Figure 6. A Cell-Intrinsic Role for Foxo1 in Control of IL-7R Expression

(A–F) Chimeras were generated by reconstitution of *Rag1*$^{-/-}$ recipients with CD45.1^{+} wild-type (WT), CD45.2^{+} CD4-Cre-Foxo1/Foxo1 (knockout, KO), or 1:1 mixed WT and KO bone marrow cells. The chimeric mice were analyzed 8 weeks after the bone marrow transfer. These are representative results of three independent experiments.

(A) Development of inflammatory bowel disease in KO chimeric mice. Hematoxylin and eosin staining of the colons of WT, KO, or mixed chimeric mice. These are representative results of four mice per group analyzed.

(B) Expression of CD44 and CD62L in splenic CD4^{+} and CD8^{+} T cells from WT, KO, or mixed chimeras.

(C) Percentage of CD4^{+}Foxp3^{+} regulatory T cells in the spleens of WT, KO, or mixed chimeras.

(D) Percentage of WT (CD45.1^{+}) and KO (CD45.2^{+}) T cells in the thymus, spleen, and peripheral lymph nodes (pLN) of a mixed chimeric mouse.

(E) Percentage of WT and KO T cells in the spleens and pLNs of the mixed chimeras that are normalized to thymic T cells (mean and SD of eight mixed chimeric mice). The p values of percentage between the two groups of mice are less than 10^{-7}.

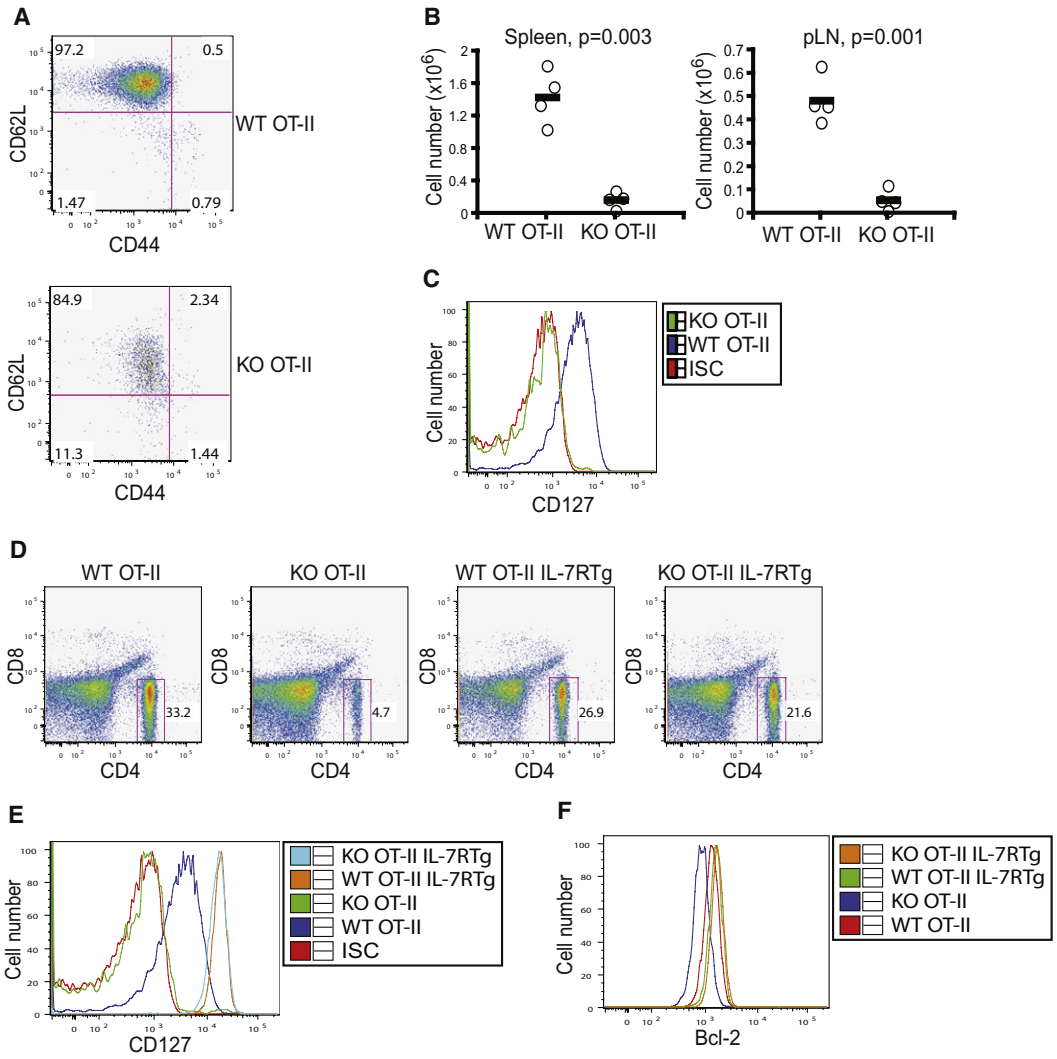

Figure 7. Foxo1 Control of IL-7R Expression and Homeostasis of OT-II T Cells

(A) Expression of CD44 and CD62L in splenic OT-II T cells from Foxo1/Foxo1 (wild-type, WT) and CD4Cre-Foxo1/Foxo1 (knockout, KO) mice at 8 weeks old. These are representative results of three independent experiments.

(B) Number of splenic and lymph node (pLN) OT-II T cells from WT and KO mice aged between 8 and 12 weeks. The p values of cell number between the two groups of mice are shown.

(C) Expression of CD127 (IL-7R) in splenic OT-II T cells from WT and KO mice. These are representative results of three independent experiments. ISC stands for the isotype control antibody.

(D) Percentage of splenic OT-II T cells from WT, KO, WT IL-7R transgenic (IL-7RTg), and KO IL-7RTg mice at 8 weeks old. These are representative results of three independent experiments.

(E) CD127 expression in splenic OT-II T cells from WT, KO, WT IL-7RTg, and KO IL-7RTg mice at 8 weeks old.

(F) Bcl-2 expression in splenic OT-II T cells from WT, KO, WT IL-7RTg, and KO IL-7RTg mice at 8 weeks old.

with LSR II (Becton Dickinson) and the FlowJo software (Tree Star). Antibodies against cell-surface markers and Foxp3 were obtained from eBiosciences. For intracellular cytokine staining, single-cell suspensions of spleens, peripheral lymph nodes (pLN), and mesenteric lymph nodes (mLN) were stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma), 1 μM ionomycin (Sigma), and GolgiStop (BD Biosciences) for 4 hr. After stimulation, cells were

(F) Expression of CD127 (IL-7R) in splenic naive ($CD44^{lo}CD62L^{hi}$) $CD4^+$ and $CD8^+$ T cells from WT, KO, or mixed chimeras. Expression of CD127 in T cells from WT and KO chimeras was plotted (left). Expression of CD127 in WT and KO T cell populations from the same mixed chimeric mouse was plotted (right). ISC stands for the isotype control antibody.

(G) Alignment of the conserved Foxo1-binding sites (FBS) in mouse and human *Il7r* gene promoter regions. The consensus Foxo1-binding sequences were marked in red. The nucleotide numbers are in reference to the translation start site (ATG, A is +1). Asterisks show the conserved nucleotides.

(H) Chromatin immunoprecipitation analysis of Foxo1 binding to the mouse *Il7r* promoter region. Immunoprecipitates from T cells were analyzed by quantitative PCR. The results were presented as fold of template enrichment in immunoprecipitates of Foxo1 antibody relative to those of an isotype control antibody (mean and SD of triplicate immunoprecipitations).

first stained with cell-surface marker antibodies, fixed and permeabilized with a Cytofix/Cytoperm kit (BD Biosciences), and stained with IFN-γ and IL-4, or IL-10 and IL-17 antibodies. Intracellular Foxp3 staining was carried out with a kit from the eBiosciences. Intracellular Ki-67 staining was performed with a kit from BD Biosciences. To analyze cell apoptosis, FACS-sorted naive T cells were cultured in the absence or presence of IL-7 for 24 hr and were stained with FITC-labeled annexin V (BD Biosciences) according to the manufacturer's instructions.

ELISA

The amounts of dsDNA and nuclear antibodies in mouse sera were determined with an ELISA kit from Alpha Diagnostic International. Sera from six pairs of WT and Foxo1-deficient mice aged 5–6 months were assayed individually with 1:100 dilution in 1% BSA PBS.

Gene Expression Profiling

Splenic and LN $CD44^{lo}CD62L^{hi}CD25^{-}$ naive $CD4^{+}$ and $CD8^{+}$ T cells from WT and Foxo1-deficient mice at 6 to 8 weeks old were purified by FACS-sorting and lyzed in QIAzol reagent. RNA was isolated with miRNeasy Mini Kit according to the manufacturer's instructions (QIAGEN). Two rounds of RNA amplification, labeling, and hybridization to M430 2.0 chips (Affymetrix) were carried out at the Core Facility of Memorial Sloan-Kettering Cancer Center. All data analyses were done with R Console. The genes with 2-fold or more change of expression were considered as Foxo1-dependent genes. The Foxo1-dependent genes shared by $CD4^{+}$ and $CD8^{+}$ T cells were divided into four categories of cell-surface proteins, signal transduction molecules, nuclear factors, and protein involved in metabolism by Gene Ontology analysis at website of David Bioinformatics Resource (http://david.abcc.ncifcrf.gov/). The heat maps were made with R Console.

Treg Cell Suppression Assay

$CD4^{+}Foxp3^{+}$ regulatory T cells (Treg) were isolated from WT and Foxo1-deficient mice that were bred to the Foxp3-RFP background by FACS sorting of $CD4^{+}RFP^{+}$ cells. $CD44^{lo}CD4^{+}RFP^{-}$ cells sorted from WT mice were labeled with 4 μM of Carboxyfluorescein diacetate succinimidyl ester (CFSE, Sigma) at 37°C for 10 min, and used as responder T cells (Tresp). 5×10^4 Tresp cells were cultured in 96-well plates with 10^5 irradiated splenocytes and 2 μg/ml CD3 antibody in the absence or presence of 5×10^4 Treg cells for 72 hr. CFSE dilution was analyzed by FACS.

Adoptive Transfer of T Cells

Splenic and LN $CD44^{lo}CD62L^{hi}CD25^{-}$ naive $CD4^{+}$ and $CD8^{+}$ T cells from 6- to 8-week-old $CD45.1^{+}$ congenic C57BL/6 (WT) mice or $CD45.2^{+}$ Foxo1-deficient mice were purified by FACS sorting and were labeled with CFSE. 2×10^6 1:1 mixed WT and Foxo1-deficient T cells were injected i.v. to 6- to 8-week-old *Rag1*$^{-/-}$ mice. Mice were sacrificed 7 days after the transfer. CFSE dilution of WT and Foxo1-deficient T cells in spleens and pLNs were determined by FACS.

Generation of Bone Marrow Chimeras

Bone marrow cells isolated from 6- to 8-week-old $CD45.1^{+}$ congenic C57BL/6 (WT) mice or $CD45.2^{+}$ Foxo1-deficient mice were depleted of erythrocytes by hypotonic lysis and of T cells and antigen-presenting cells by complement-mediated cell lysis. 2×10^6 WT, Foxo1-deficient, or 1:1 mixed WT and Foxo1-deficient bone marrow cells were injected i.v. to 6- to 8-week-old *Rag1*$^{-/-}$ mice that were sublethally irradiated (600 rad).

Statistical Analyses

Student's t test was used to calculate statistical significance for difference in a particular measurement between groups. $p < 0.05$ was considered statistically significant.

ACCESSION NUMBERS

The expression data have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (GSE15037).

SUPPLEMENTAL DATA

Supplemental Data include seven figures and three tables can be found with this article online at http://www.immunity.com/supplemental/S1074-7613(09)00107-1.

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