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FORUM REVIEW ARTICLE

Highly Specialized Role of Forkhead Box O Transcription Factors in the Immune System

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Abstract

Recent studies have highlighted a fundamental role for Forkhead box O (Foxo) transcription factors in immune system homeostasis. Initial reports designed to dissect function of individual Foxo isoforms in the immune system were based on *in vitro* overexpression systems, and these experiments suggested that Foxo1 and Foxo3 are important for growth factor withdrawal-induced cell death. Moreover, Foxo factors importantly regulate basic cell cycle progression, and so the implication was that these factors may control lymphocyte homeostasis, including a critical function in the termination and resolution of an immune response. Most recently, cell-type-specific loss mutants for the different Foxo isoforms have revealed unexpected and highly specialized functions in the control of multiple cell types in the immune system, but they have yet to reveal a role in cell death or proliferation. This review will focus on the recent advances made in the understanding of the many ways that Foxo factors regulate the immune system, including a discussion of how the specialized *versus* redundant functions of Foxo transcription factors impact immune system homeostasis. *Antioxid. Redox Signal.* 14, 663–674.

Introduction

IMMUNE SYSTEM HOMEOSTASIS is regulated by a multitude of mechanisms allowing the development of responses to pathogens while avoiding attacks to innocuous, commensal, or self-antigens. Further, the response to pathogens is also tightly controlled: activation, division, and differentiation allow the generation of a large pool of antigen-specific cells harboring effector properties appropriate for the type of pathogen encountered. After pathogen clearance, there is a contraction phase where the majority of cells are eliminated to allow the system to return to a predetermined number of cells; meanwhile, some cells survive apoptotic death to become memory cells. Perturbations in immune cells life-or-death balance may lead to pathological processes, such as immunodeficiency, autoimmunity, or lymphoproliferative disorders, and factors responsible for the dysregulation of the immune system remain largely unknown. Identification of such factors is of prime interest to decipher pathways involved in immune-mediated diseases. In this regard, Forkhead box O (Foxo) transcription factors appear to be particularly important in sensing the environmental stimuli such as nutrients, growth factors, or stress, and in converting this information into a program of gene expression dictating proliferation, differentiation, survival, or death in many different cell types.

Foxo proteins are mammalian homologs of DAF-16, identified in *C. elegans* as a major regulator of lifespan and stress resistance (46). They belong to the Forkhead box family of transcription factors characterized by a conserved winged helix DNA binding domain called the forkhead domain (57, 78). In mammals, the Foxo subclass is comprised of four members: Foxo1 (FKHR), Foxo3 (FKHRL1), Foxo4 (AFX), and Foxo6. Foxo6 expression is confined to specific region of the brain (42), whereas Foxo1, 3, and 4 are ubiquitously expressed, but between different cell types and organs, a heterogeneous pattern of expression has been described (29) (Novartis Gene Expression Atlas). Foxo1 and Foxo3 are the main isoforms expressed in the immune system, but their expression levels differ between organs of the immune system and between lymphoid and myeloid cell types: Foxo1 expression is higher in spleen and lymph node as compared with Foxo3, which is the main transcript detected in the thymus and bone marrow (Fig. 1A and unpublished data). Within the spleen, Foxo1 is predominantly expressed in T cells and B cells, whereas Foxo3 is mainly expressed in granulocytes, macrophages, and dendritic cells (DCs) (Fig. 1B and unpublished data). Regulation of Foxo transcriptional activity is complex and mainly dependent

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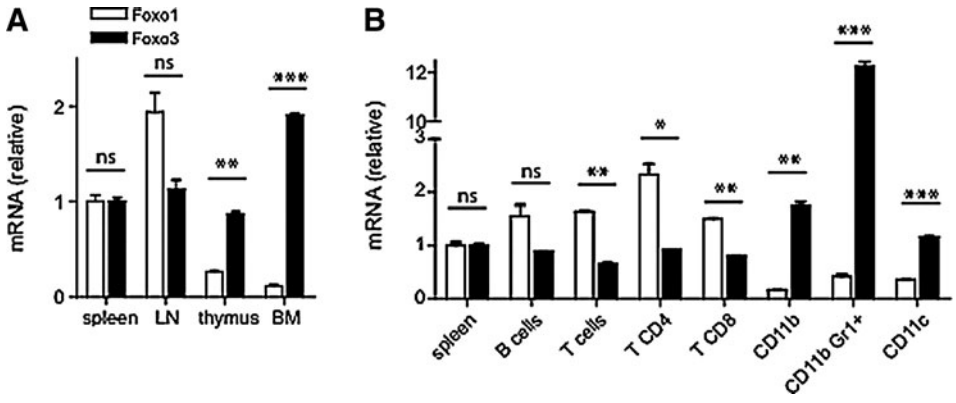

FIG. 1. Expression pattern of Foxo1 and Foxo3 in the immune system. (A) Quantitative polymerase chain reaction analysis of Foxo1 and Foxo3 mRNA expression in tissues from C57BL/6 mice. **(B)** Quantitative polymerase chain reaction analysis of Foxo1 and Foxo3 mRNA expression in purified cell subsets from C57BL/6 mice spleen. The abundance of Foxo1 and Foxo3 mRNA in each sample was normalized to that of Hprt1 mRNA and then normalized to the amount obtained for the spleen (set to 1). Data in **A** and **B** are mean plus/minus SD of duplicate samples. Results are representative of two independent experiments. $*p < 0.01$; $**p < 0.005$; and $***p < 0.001$ (unpaired two-tailed Student's *t*-test) (unpublished data). BM, bone marrow; Foxo1, Forkhead box O transcription factor; LN, lymph node; ns, not significant.

on posttranslational modifications that affect Foxo subcellular localization and includes phosphorylation, acetylation, ubiquitination, methylation, and O-linked glycosylation (11). Depending on the stimuli, these modifications actively determine nuclear *versus* cytoplasmic localization.

Binding of growth factors, including insulin-like growth factors or insulin, to their receptors initiates phosphatidylinositol 3-kinase (PI3K) and Akt (serine/threonine protein kinase, also known as protein kinase B [PKB]) activation, followed by Foxo phosphorylation. Phosphorylation of Foxo proteins elicits both cytoplasmic sequestration and the degradation of Foxo proteins (10, 41). Foxos are therefore the major downstream target of the PI3K/Akt signaling pathway, implying that Foxo proteins might control any process sensitive to the PI3K pathway in the sense of negative regulation (Fig. 2). Indeed, in immune cells, the PI3K pathway is activated by different stimuli *via* specific receptors, including the B cell antigen receptor (BCR), T cell antigen receptor (TCR), cytokine, and chemokines receptors but also toll-like receptors (TLRs), and it plays a crucial role in immune cell functions (27, 72) [for review see (17)]. It is also involved in T and B cell development since mice deficient for the p85α regulatory or p110δ catalytic subunit of the PI3K exhibited severe impaired B and T cell development and functions (27, 72). The PI3K pathway also has broad and distinct roles in innate immune cells, including neutrophils, mast cells, monocytes, macrophages, and DCs. PI3Kc-deficient neutrophils exhibit severe defects in migration (48, 67) and phagocytosis (36). In mast cells, inactivation of the p110δ isoform of PI3K leads to defective proliferation, adhesion, and migration and impaired degranulation and cytokine release (1). PI3Kδ is critical for type I interferon production by plasmacytoid DCs (35). Finally, in monocytes, macrophages, and myeloid DCs, PI3K is involved in TLR signaling and may limit proinflammatory cytokine production such as interleukin (IL)-12 while enhancing antiinflammatory IL-10 synthesis (28, 34).

Stress stimuli can override presence of growth factors by triggering Foxo nuclear relocalization and activation. Oxidative stress results in JNK-dependent phosphorylation of Foxo proteins, nuclear translocation, and transcriptional activation

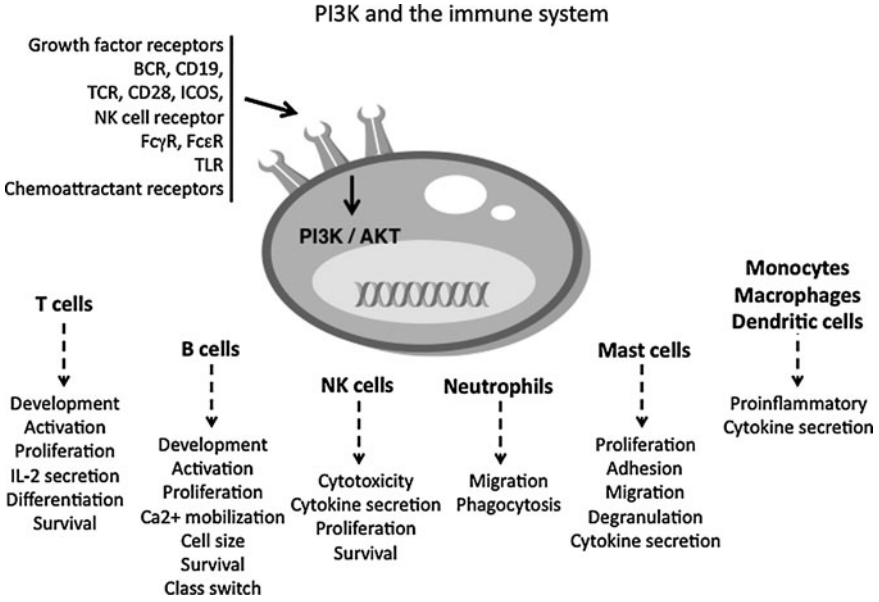

FIG. 2. PI3K in immune system. The PI3K signaling pathway is activated *via* specific receptors, including the BCR and CD19 expressed by B cells, TCR, CD28, or ICOS expressed on T cells, NK cell receptors but also cytokine and chemokine receptors and TLRs. This pathway regulates many cellular process involved in immune cells development, survival, proliferation, and functions. Akt, serine/threonine protein kinase, also known as protein kinase B; BCR, B cell antigen receptor; ICOS, inducible costimulator; IL, interleukin; NK cells, natural killer cells; PI3K, phosphatidylinositol 3-kinase; TCR, T cell antigen receptor; TLR, toll-like receptor.

of Foxo (22). Immune cells are highly sensitive to oxidative stress: reactive oxygen species effectively promote the elimination of antigen-specific activated T cells (20, 38). Intracellular redox status also regulates T cell activation and apoptosis during normal T cell development in the thymus, and during immune response in peripheral lymphoid organs (59). In this regard, an aberrant increase of reactive oxygen species in Foxo1/3/4-deficient mice was observed in the hematopoietic stem cell (HSC) compartment associated with a defect in cell cycle progression and an increased apoptosis. Treatment of deficient mice with an antioxidant, *N*-acetyl-L-cysteine, restored the size of the HSC compartment. Thus, Foxos function in the HSC compartment is to maintain cell quiescence by controlling response to physiologic oxidative stress (74).

Foxo function is also dependent on metabolism availability. Under conditions of calorie restriction, AMPK is required to extend lifespan and delay age-dependent decline in *C. elegans*. AMPK activates Foxo-dependent transcription and phosphorylates Foxo/DAF-16, suggesting a possible direct mechanism of regulation of Foxo/DAF-16 by AMPK (31). A new mechanism of cross-regulation between metabolism and innate immunity that involved Foxo has been recently reported. Foxo factors are involved in the antimicrobial peptide gene activation, the first line of defense against opportunistic and pathogenic bacteria. Foxo-dependent regulation of antimicrobial peptides is evolutionarily conserved from *Drosophila* to mammals and can be activated under normal physiological conditions in response to starvation. Thus, these data provide evidence of how Foxo factors are crucial for the adaptation of organismal defence to growth factor availibility (6). The ability of a lymphocyte to stay quiescent, proliferate, or die is also controlled by its ability to collect the nutrients that are required to support cell growth therefore tight regulation of glucose uptake is required to maintain immune homeostasis. Changes in glucose metabolism might affect cell death pathways and be crucial in the development, selection, and function of lymphocytes (43).

Finally, to add another degree of complexity to an already very intricate network, Foxo transcriptional function is also dictated by protein–protein interactions. Indeed, Foxo can regulate transcriptional responses independently of direct DNA binding. Foxos can associate with a variety of unrelated transcription factors, including Smad, STAT, PPAR, Runx, or P53. So far, more than 20 cofactors have been identified with diverse implications within the immune system [for review see (75)]. Among these factors, Smad proteins are probably the most likely to play a key role in immune cell homeostasis. Smad proteins belong to the transforming growth factor (TGF)β signaling pathway and coimmunoprecipitation experiments confirmed that Foxo proteins bind to Smad3 and Smad4 in a TGFβ-dependent manner (70). Active TGFβ signals through two trans-membrane serine-threonine kinase receptors, TGFβ receptor I and II (TGFβRI and TGFβRII) (55). Activated TGFβR phosphorylates the transcription factors Smad2 and Smad3, triggering their translocation into the nucleus assocated with Smad4. Smad complexes, in association with additional transcription factors, bind to the regulatory sequences in target genes and regulate gene expression. These complexes target different genes, depending on their composition (56).

Physical interaction of Smad4 with Foxo protein has an impact on the TGFβ cytostatic functions. Seoane and colleagues showed that Foxo1, Foxo3, and Foxo4 bind specifically and directly to Smad3 and Smad4, and contribute to the TGFβ-driven program of gene expression. The use of a dominant-negative Foxo construct inhibited $\sim$15 out of the 118 TGFβ early response genes in a keratinocyte (HaCaT) cell line. This study also confirmed that Smad/Foxo complexes target the region of the $p21^{CIP1}$ promoter containing a Forkhead binding element and a SMAD binding element resulting in transactivation of p21Cip1, a cyclin-dependent kinase inhibitor involved in cell cycle arrest (70).

TGFβ signaling is also crucial for immune cell homeostasis and T cell tolerance *in vivo*, since mice harboring a T cell-specific deletion of the TGFβRII developed a lethal autoimmune syndrome characterized by heavy infiltration of leukocytes into multiple tissues including stomach, lung, liver, pancreatic islets, and thyroid. A defect in TGFβ signaling led to Tbet upregulation by CD4 T cells, uncontrolled differentiation into Th1 cells, and hyperproliferation leading to a florid autoimmune syndrome (52). Moreover, TGFβ drives expression of Foxp3, the master gene for regulatory T (Treg) cell development and functions (82), and is essential for the survival of peripheral Treg cells *in vivo*. Whether Foxo proteins are involved in these processes has to be further analyzed.

Because of the shared DNA-binding domain, Foxos are expected to bind to similar sequences and therefore are likely to regulate the same set of target genes (29) (7); however, a growing number of studies have begun to elucidate the role of individual Foxo members in immune homeostasis using genetically modified mice. Despite overlapping tissue expression, and the apparent redundancy of function *in vitro*, genetic loss of the different Foxo isoforms does result in specific phenotypes and thus highlighting the independent physiological functions for various Foxo proteins.

Forkhead Box O Transcription Factor 3

Initial reports designed to understand the role of Foxo3 in immune system homeostasis were based on *in vitro* overexpression experiments, and these experiments suggested a role for Foxo3 in T and B cell death. In particular, Foxo3 appeared to be required for activation-induced cell death, a Fas-dependent type of apoptosis, or growth factor deprivation-induced apoptosis, both of which have been proposed as mechanisms important for the contraction phase of an immune response (Fig. 3).

Transfection of Jurkat T cells with a nonphosphorylable mutant form of Foxo3-induced T cell apoptosis by a Fas ligand-dependent mechanism. In fibroblasts, the presence of the survival factor triggered Foxo3 phosphorylation resulting in its interaction with the adaptor protein 14-3-3. This, in turn, was responsible for Foxo3 nuclear export and inhibition of its transcriptional activities, and thus served to protect these cells from apoptosis (9). Phosphorylation and inactivation of Foxo3 appears to play an important role in IL-2-mediated T cell survival. Optimal activation of T lymphocytes requires TCR complex engagement, accompanied by a costimulatory signal that can be provided by either CD28 or IL-2 (51). IL-2 is a potent mitogen for T cells that promotes proliferation and survival of activated T cells through the PI3K signaling pathway. In murine CTLL-2 T lymphocytes, IL-2 withdrawal resulted in G1 phase cell cycle arrest followed by apoptosis, and these events correlated with Foxo3 activation. Foxo3

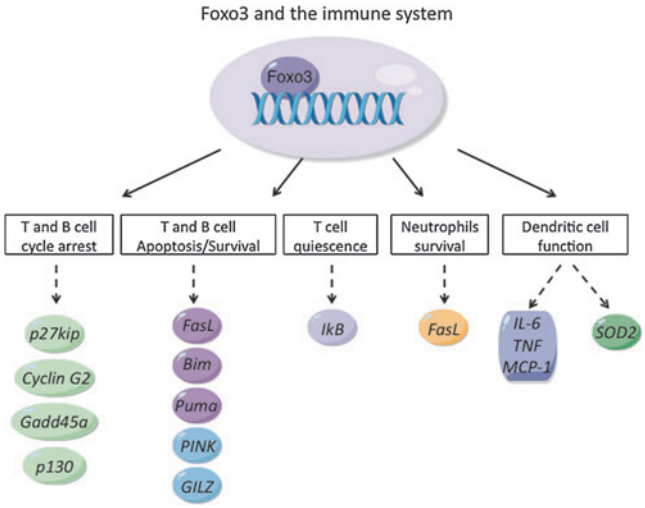

FIG. 3. Roles of Foxo3 transcription factors in the immune system. Foxo3 transcription factors trigger a variety of cellular processes by regulating target genes involved in T and B cells survival, proliferation, and death, neutrophils survival, and DCs stress resistance and cytokine secretion. DC, dendritic cell; MCP1, monocyte chemotactic protein-1; SOD, superoxide dismutase; TNF, tumor necrosis factor. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

appears to be responsible for expression of the cyclin-dependent kinase inhibitor $p27^{KIP1}$ and the proapoptotic Bcl-2 family member Bim. Bim and $p27^{KIP1}$ were downregulated in a PI3K-dependent fashion in response to IL-2 (71). Moreover, expression of a mutant form of Foxo3 confined to the nucleus was sufficient to mimic the effects of IL-2 withdrawal. Activation of Foxo3 in response of IL-2 deprivation has also been demonstrated in primary T cells. In these cells, chromatin immunoprecipitation assays confirmed the physical interaction between endogenous Foxo3 and the Bim and Puma promoters after IL-2 withdrawal (80). The model proposed is that in the absence of IL-2, as PI3K/Akt signaling is downregulated, Foxo3 is activated to induce the proapoptotic factors, Puma and Bim.

Paradoxically, some Foxo3 target genes induced in response of cytokine starvation exhibit antiapoptotic function. For example, the phosphatase and tensin homolog-induced putative kinase 1 was described as an antiapoptotic Foxo3 target gene whose induction upon growth factor deprivation prolongs lymphocyte survival (58). Similar observations have been made for the Glucocorticoid-induced leucine zipper (GILZ) that is upregulated upon IL-2 withdrawal, inducing a delay in IL-2 withdrawal-mediated apoptosis. The presence of two Forkhead responsive elements in the Gilz promoter suggests a direct Foxo3-dependent transcriptional control of this gene (3). GILZ-dependent T cell death protection seems to be due, at least in part, to the inhibition of Bim and $p27^{KIP1}$ expression. GILZ might act as a general inhibitor of FOXO factors by promoting relocalization of Foxo protein to the cytoplasm and thereby inhibiting its transcriptional activity (49). Hence, activation of Foxo3 within T cells seems crucial for the control of T cell death/survival balance in response to growth factor deprivation or cytokine withdrawal and may participate in the termination of the immune response.

Memory T cell survival is also dependent upon the activity of Foxo3. Foxo3 upregulation is associated with impaired memory T cell survival in a mouse model of viral *versus* bacterial infection (79). Foxo3 activity controls the difference in survival capacity between central memory (Tcm) and effector memory (Tem) T cells in human. Gamma-chain receptor-cytokines such as IL-2 or IL-7 play a fundamental role in the differentiation and the maintenance of memory T cells. TCR-mediated signaling combined with either IL-2 and IL-7 signaling synergistically induced Foxo3 phosphorylation, thereby preventing the transcription of FasL, Bim, Gadd45a, and p130 proapoptotic molecules, and this provided Tcm cells with the capacity to survive and resist apoptotic signals. Inactivation of Foxo3 in Tcm cells required I-kappaB kinase and AKT, since AKT and I-kappaB kinase inhibitors prevented Foxo3 phosphorylation and increased expression of proapoptotic molecules leading to apoptosis (66). The pathophysiological relevance of this finding has been demonstrated in HIV^+ elite controller (EC) subjects. These patients demonstrated functional memory responses able to control HIV replication for more than 9 months in the absence of therapy. Tcm as well as Tem purified from EC patient were refractory to death when compared with T cells from HIV aviremic, successfully treated subjects or uninfected controls. This increased survival appeared to be due to lower expression of Fas and higher expression of Bim and p130 in both Tem and Tcm from EC patients rendering these cells less susceptible to apoptosis. This increased survival has been associated with increased Foxo3 phosphorylation. Moreover, silencing of Foxo3 in memory $CD4^+$ T cells from successfully treated patients using specific small interfering RNAs elicited a 70%–80% downregulation of Bim and p130, protection against Fas-induced apoptosis, and an increased survival of memory cells to an extent similar to cells from EC subjects. Foxo3 is thus involved in the increased susceptibility of memory T cells to apoptosis during HIV infection, and strategies to inhibit Foxo3 transcriptional functions may have an important role in future drug discovery in the treatment of chronic infections (76).

A similar pathway has been described for B cells where the main function of Foxo3 would be to limit B cell proliferation and survival after BCR engagement. In mouse primary B cells, forced expression of AKT-independent variants of Foxo3-induced partial arrest in G1 phase of the cell cycle and increased apoptosis. *Ccng2* (cyclin G2) and *Rbl2* (p130) genes, implicated in cell cycle arrest, were identified as candidate Foxo target genes important for B cell quiescence (81). Foxo3 (as Foxo1) may act in synergy with the zinc finger transcription factor δEF1 to specifically enhance the transcription of both of these genes (15). In the mouse pre-B cell line Ba/F3, which requires IL-3 both for proliferation and survival, cytokine withdrawal induced both arrest in G1 and apoptosis, phenotypes that can be mimicked by expression of a constitutively active mutant of Foxo3. Cell cycle arrest is due to Foxo3-dependent expression of $p27^{KIP1}$ and the subsequent apoptosis to the upregulation of Bim (21). Moreover, in BCR/Abelson (Abl) tyrosine kinase (ABL)-expressing BaF3 cells, regulation of cyclin D2 is dependent on Foxo3 and involves induction of the transcriptional repressor Bcl-6. BCR-ABL inhibition leads to activation of Foxo3 and expression of Bcl6,

and this is responsible for the repression of cyclin D2 (26). Thus Foxo3 inactivation is a functionally important consequence of PI3K signaling in primary B cells. Moreover, generation of long-term memory B cells is also dependent on expression of Bcl-6 (73).

The message from these *in vitro* studies is that Foxo3 may be involved during the contraction phase of the immune response when growth factors and cytokines are diminished. It appears to induce death effector molecules such as Fas, Bim, or Puma as well as cell cycle inhibitors such as $p27^{KIP1}$ and p130 to limit proliferation and survival of effector and memory cells. Foxo3 may also function in the termination of an immune response to constrain T cell viability and allow the system to return to a baseline number of cells. However, the characterization of Foxo3-deficient mice revealed more complex physiological functions for this transcription factor that were not predicted by the Foxo3 expression patterns nor the conclusions drawn from the *in vitro* overexpression systems described above.

The first report on Foxo3 null mice that specifically addressed the immune system came from Lin *et al.* (53). Mice used in this report were created by retroviral gene-trap techniques using embryonic stem cells from strain 129 mice ($Foxo3^{Trap}$ mice). Early development of these mice appeared normal although the authors reported female infertility due to abnormal ovarian follicular development as previously reported for other Foxo3 null strains (13, 40). However, at 16 weeks of age $Foxo3^{Trap}$ mice developed a mild, apparently nonlethal inflammation predominantly affecting the salivary glands. Infiltration of $CD4^+$ and $B220^+$ lymphocytes were detected in the salivary glands, lungs, and kidneys associated with a lymphoproliferative syndrome, mainly consisting of T cells in the lymph nodes and spleen. This autoimmune syndrome was likely due to a defect in the regulation of T cell proliferation or activation rather than a defect in cell death since the authors failed to identify any apoptosis defect for $Foxo3^{Trap}$ T cells and B cells. $Foxo3^{Trap}$ animals did not demonstrate significant hypergammaglobulinemia or autoantibody production (including antinuclear antibodies, rheumatoid factor, or anti-dsDNA). Th1 and Th2 hyperactivation was proposed to be caused by constitutive nuclear factor-kappa B (NF-κB) activity as measured by decreased IκBβ and IκBε expression and correlated with decreased expression of Foxj1. Blockade of NF-κB activity appeared to diminish this hyperactivity; however, the authors failed to identify any direct targets of Foxo3 that could account for repression of NF-κB activity. Nonetheless, this study was interpreted to support a role of Foxo3 in maintaining T cell tolerance and quiescence.

Other studies, including ours using two different Foxo3-deficient mouse strains, did not find immunological abnormalities, including inflammation described above (13, 18, 40). One mouse strain, $Foxo3^{Kca}$, also generated as part of a retroviral gene-trap project (Lexicon Genetics, Inc.) was backcrossed to the C57BL/6 strain. The second strain we used, $Foxo3^{-/-}$ mice, were generated by targeted recombination and maintained as congenic FVB mice. Neither of these mouse strains exhibited signs of spontaneous autoimmunity as assessed by histological analysis (13, 40) or detailed enumeration of T and B cells (18). The number and proportion of activated or effector-memory T cells in spleen and lymph node from naive mice were not affected by Foxo3 deficiency. Moreover, T cells purified from $Foxo3^{Kca}$ and $Foxo3^{-/-}$ mice showed no defect in proliferation and survival after *in vitro* stimulation and no change in expression of IκBα, IκBβ, or IκBε protein relative to that of wild-type T cells. These results suggest that a loss of Foxo3 alone is not sufficient to elicit manifestations of T cell activation or autoimmunity.

There is, however, an important role for Foxo3 in a T cell response to viral infection. When $Foxo3^{Kca}$ mice were infected with lymphocytic choriomeningitis virus (LCMV) and antigen-specific T cell populations were enumerated, we observed a marked increased T cell expansion as compared with wild-type (WT) mice. The kinetics of the immune response were unchanged in $Foxo3^{Kca}$ mice although LCMV-specific CD4 and CD8 T cell populations were increased more than threefold. This increased T cell accumulation observed in Foxo3-deficient mice was not specific to the response to LCMV since these results were reproduced with vesicular stomatitis virus. Analysis of bone marrow chimeras and adoptive transfer experiments revealed that the phenotype is intrinsic to the hematopoietic cell compartment but, surprisingly, does not depend on T cells.

This strongly suggested a requirement for Foxo3 in innate immune cells. We noted a greater proportion of $Gr\text{-}1^{high}$ $CD11b^{high}$ cells (which include granulocytes and macrophages) and erythrocyte progenitors (Ter119+; data not shown) as reported before (54). Examination of the number, phenotype, or function of DCs revealed that Foxo3 display a unique, nonredundant role in these cells. We reported a highly significant increase in the number of DCs in naive $Foxo3^{Kca}$ mice associated with an enhanced maturation state. DCs from *Foxo3*-deficient mice exhibited an increased ability to stimulate T cells through an enhancement of T cell survival. This function is cell-autonomous since DCs generated *in vitro* from bone marrow cells (BMDCs) cultivated in presence of GM-CSF more effectively sustained T cell viability when compared with WT BMDCs. This increased T cell survival was found to be dependent on IL-6 secretion by Foxo3-deficient DCs. DCs from Foxo3-deficient mice exhibited enhanced production of IL-6, tumor necrosis factor, and monocyte chemotactic protein-1 (MCP-1), implying a master role for Foxo3 in regulating proinflammatory cytokine production. Among these cytokines, we showed that IL-6 played a major role. Treatment of mice with an anti-IL-6Rα blocking Abs substantially and specifically reduced the magnitude of LCMV T cells immune response in Foxo3-deficient mice, but this treatment did not affect the number of LCMV-specific T cells in wild-type mice.

An important concept is that Foxo3 may be involved in the bi-directional signaling that occurs between CD28 or cytotoxic T-lymphocyte antigen 4 (CTLA4) expressed on T cells and their counter-receptors expressed by DC, B7-1, or B7-2. These signaling pathways modulate T cell survival by affecting the DC production of stimulatory or suppressive molecules. Stimulation through CD28 promotes T cell activation, whereas B7 signaling promotes either IL-6 or interferon γ production by DCs (61) (Fig. 4A). However, stimulation of $Foxo3^{Kca}$ DCs with a soluble form of CD28 induced a similar IL-6 production to that of WT DC (data not shown), suggesting that B7 signaling through CD28 is not affected by the loss of Foxo3 and is not involved in the difference in IL-6 production observed between wildtype and $Foxo3^{Kca}$. CTLA4 acts as a counterbalance to CD28 costimulation, antagonizing TCR signals and promoting tolerance (45). Stimulation through CTLA4 inhibits IL-2 production and T cell proliferation (30, 77). Moreover,

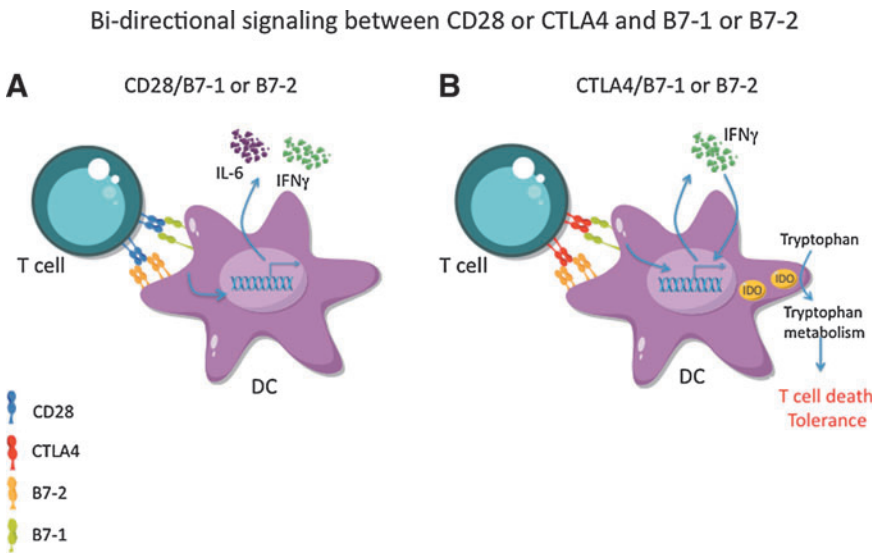

FIG. 4. Bi-directional signaling between CD28 or CTLA4 and B7-1 or B7-2. (A) CD28 engagement of B7 molecules on DCs results in concomitant secretion of both IL-6 and IFNγ. **(B)** CTLA-4 engagement of B7 molecules on DCs stimulates IFNγ production, which promotes upregulation of IDO, an enzyme involved in tryptophan catabolizes and leads to inhibition of T cell responses. IDO, indoleamine 2,3-dioxygenase; IFN, interferon. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

upregulation of CTLA-4 on preactivated T cells correlated with the upregulation of Bcl-2 through the inhibitory phosphorylation of Foxo3, and this in turn prevented Fas/FasL expression. Thus, the PI3K/Foxo3 pathway acts to rescue T helper cells from apoptosis induced by CTLA-4 (64). The reverse signaling induced by CTLA4 ligation of B7 is known to activate the immunosuppressive pathway of tryptophan catabolism in DCs involving indoleamine 2,3-dioxygenase (IDO) (32) (Fig. 4B). It has been suggested that Foxo3 has a role in IDO pathway induced by CTLA4 ligation on DCs since CTLA4–immunoglobulin (Ig) leads to Foxo3 nuclear accumulation and superoxide dismutase 2 (SOD2) expression. Consistent with this, a knockdown of Foxo3 was shown to inhibit tryptophan catabolism (25). Since IDO is known to induce T cell death through the generation of kinurenin and tryptophan privation (32), we speculated that a potential IDO defect in *Foxo3*^{Kca} DCs may explain the increased T cell survival; however, the addition of 1-methyl-d-tryptophan IDO inhibitor to cultures did not enhance T cell survival in cultures containing wildtype or *Foxo3*^{Kca} BMDCs (data not show). Instead of activating IDO immunosuppressive pathway, these data suggest that CTLA4 modulates wildtype DCs function through a Foxo3-dependent inhibition of IL-6 and tumor necrosis factor production. Upon TLR stimulation, Foxo3 remained inactive in the cytoplasm, whereas simultaneous stimulation using CTLA4-Ig promoted Foxo3 dominant nuclear accumulation (18). Altogether, these experiments indicate nuclear-localized Foxo3 functions in DCs to inhibit the production of IL-6 and other inflammatory cytokines that would otherwise be activated in response to infection. Since activated T cells express CTLA4, we deduce that the IL-6-induced T cell survival found in *Foxo3*^{Kca} mice results from an inability of Foxo3-deficient DCs to shut down cytokine production in response to the CTLA4 signal (Fig. 5). Finally, our results highlight an unappreciated role of activated T cells in the control of DC functions and immune response magnitude.

Thus, contrary to what was expected, T cells are not obviously affected by the loss of Foxo3. Foxo3 by itself appears to be dispensable for T cells homeostasis *in vivo* although other Foxo isoforms, particularly Foxo1, may act in to compensate in the absence of Foxo3. Instead, a critical function for Foxo3 in the control of T cell responses that is confined to DCs has been revealed emphasizing that the main nonredundant function of Foxo3 in the immune system is to suppress inflammatory cytokine production by DCs. Foxo3 is likely to have important role in other cells of the innate immune. For example, in neutrophils Foxo3 is involved in migration (16) and survival in the context of inflammation (44), but there are likely to be many functions for Foxo3 in cells of the innate immune system still to be discovered.

Forkhead Box O Transcription Factor 1

Until recently, a vast majority of studies were focused on the role of Foxo3 in the immune system, whereas only a few

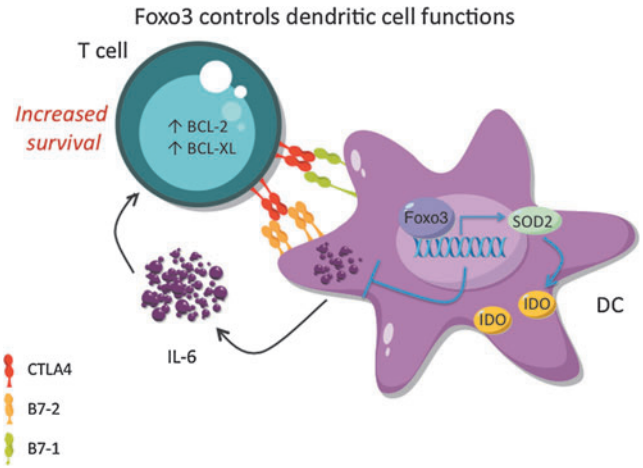

FIG. 5. Foxo3 controls DCs functions. The reverse signaling induced by CTLA4 ligation of B7 leads to Foxo3 nuclear accumulation. Once activated, Foxo3 directs SOD2 expression involved in activation of the IDO-dependent immunosuppressive pathway in DCs. In addition of controlling the redox status, Foxo3 major function in DCs is to constrain the production of IL-6 and then limit T cell survival. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

looked at a role for Foxo1. Initial expression analysis patterns showed that Foxo3 mRNA was expressed at relatively high levels in numerous organs. In contrast, Foxo1 seemed to have a more restricted pattern of expression, being predominantly detected in the ovaries, the uterus, and the adipose tissues (7, 29). This notion has been more recently challenged by our group, as well as large-scale gene expression analyses showing that in human and mice, Foxo1 mRNA expression is preferentially expressed in ovaries but also in mature peripheral T and B cells compared with many other tested tissues or purified cell populations [(47) and http://biogps.gnf.org/). As opposed to broad expression of Foxo3, this specific expression pattern and its conservation across species suggested a critical role for Foxo1 in lymphocytes.

The first *in vitro* studies on Foxo1 in T and B cells, but also more recently in DC, confirmed that Foxo1 activity follows the same regulatory mechanisms as Foxo3. Stimulation through common gamma chain cytokine receptors (γ_C), such as the IL-2 or IL-7 receptors, or through antigen receptors, either the TCR or BCR, leads to a PI3K-dependent phosphorylation and nuclear exclusion of Foxo1 (5, 14, 24, 65, 71, 81). Further, defective PI3K activation in *Vav1*$^{-/-}$ T cells or mutation of the three Akt-phosphorylation sites on Foxo1 (T24, S256, and S319) is associated with constant nuclear localization of Foxo1 in T and B cells irrespective of the cell activation status (14, 24, 81). Similar to Foxo3, defective Foxo1 inactivation or enforced Foxo1 activation has been correlated to cell cycle arrest and impaired $p27^{KIP1}$ downregulation in activated T cells, as well induction of apoptosis in LPS-stimulated B cells (14, 24, 81). Consistent with these observations, the first *in vivo* study using mice expressing a dominant negative form of Foxo1, while displaying only minor differences in T cell development, had a increase in the proliferation of each of the major thymic cell subpopulations (50). On the basis of these data and the highly specific and conserved expression pattern of Foxo1, a critical role in the active maintenance of T and B cell quiescence was proposed (81). Further, Foxo1 could also have a dominant role in growth factor withdrawal-induced cell death as part of the regulation of lymphocyte homeostasis or the termination of lymphocyte expansion.

Most of these initial studies and hypotheses were carried out based on the observation that the antiproliferative and pro-apoptotic functions of Foxo transcription factors were highly conserved across cell types and species. Until recently, these biased approaches, although confirming a role for Foxo1 in the circle of life and death of several immune cell types, did not anticipate more specialized functions of Foxo1 in lymphocyte biology. The lack of a genetic-based modulation of Foxo1 function hampered precise analyses of the role of Foxo1 in the highly intricate physiology of the immune system. Indeed, the Foxo1-null genotype in mice is embryonic lethal, and the embryos death at E10.5 prevents the generation of fetal liver chimeras (40). To investigate its role in lymphocytes more precisely, several groups recently used complementary unbiased experimental strategies that revealed unexpected and highly specialized functions for Foxo1 in T and B cells.

First, Foxo1 has been shown to control T cell homing to secondary lymphoid organs. Gene array analysis revealed that expression of a constitutively active mutant of Foxo1 in the human T cell line Jurkat resulted in expression of a set of genes involved in lymphocyte recruitment into the secondary lymphoid organs (23). Notably, Foxo1 activation induces expression of the cell adhesion lectin CD62L (L-selectin, *Sell*) involved in lymphocyte adhesion to the high endothelial venule of the lymph nodes; the chemokine receptor CCR7 responsible for T cell migration into the T cell zone of the spleen and lymph nodes, and the sphingosine-1-phosphate receptor $S1P_1$ (*Edg1*) involved in T cell egress from lymphoid organs. Concomitantly, those results have been confirmed by our group and others with the use of several models of conditional deletion of Foxo1 directly *in vivo*. Constitutive deletion of Foxo1 in the T cell lineage as well as acute deletion leads to T cell homing defects to peripheral lymphoid organs associated with downregulated expression of *Sell*, *Ccr7*, *Edg1*, as well as the transcription factor Klf2 [(33, 47, 62) and unpublished data]. Importantly, KLF2 is known to have a specialized role in the regulation of T cell trafficking through its control of *Sell* and *Edg1* expression (4, 12). Supporting the idea of an indirect role for Foxo1 in the control of *Sell* and *Edg1* expression through Klf2 regulation, chromatin immunoprecipitation assays revealed that Foxo1 directly binds to the Klf2 promoter (23). Although the precise molecular basis involved needs further investigation, the activation of Foxo1 in T cells drives their recruitment and migration into the T cell zone of secondary lymphoid organs.

In addition to the impaired T cell trafficking of Foxo1-deficient T cells, we and others have shown that Foxo1 is required to sustain naive T cell survival. Adult $Foxo1^{f/f}$ Cd4Cre mice harbor a significantly decreased population of naive T cells in both lymph nodes and spleen, associated with a failure to be maintained upon transfer to a wild-type host and decreased expression of the antiapoptotic protein Bcl2 (47, 62). Further experiments aiming to identify the molecular mechanisms underlying these defects showed that Foxo1 is necessary for expression of the receptor for the IL-7 receptor (R)α, a crucial survival factor for naive CD4 and CD8 T cells (47, 62, 68, 69). This correlates with Foxo1 binding to an enhancer located 3.5 kb upstream of the *Il7ra* gene, supporting a direct role of Foxo1 in expression of the IL-7Rα (47, 62). Together, with its role in T cell homing to secondary lymphoid organs, this supports a role for Foxo1 as a critical integrator of a safety mechanism for naive T cells. In situations of growth factor starvation, Foxo1 activation would ensure that T cells would be preferentially recruited to the lymphoid organs where the IL-7 is secreted and upregulate expression of the receptor for this critical pro-survival cytokine (Fig. 6). Importantly, similar to T cells, conditional deletion of Foxo1 at different stages in B cell development *in vivo* showed that it is necessary to maintain pro-B cell survival through the induction of IL-7Rα expression and presumably homing through expression of CD62L (19). Thus, the role of Foxo1 in the regulation of IL-7Rα expression and cell trafficking is conserved among lymphocyte populations.

In addition to its critical role in naive T cell homeostasis, it has also recently been suggested that Foxo1 would be involved in T cell tolerance. Radiation chimeras generated with bone marrow from $Foxo1^{f/f}$ Cd4Cre mice spontaneously develop colitis associated with a decreased frequency of $Foxp3^+$ Treg cells (62). This phenotype is rescued in mixed bone marrow chimeras, suggesting a defective dominant tolerance in the single reconstituted chimeras and thus a role for Foxo1 in Treg cell development and/or function. Yet, in mixed chimeras, the highly impaired competitiveness of Foxo1-deficient T cells, due to their impaired survival and migration,

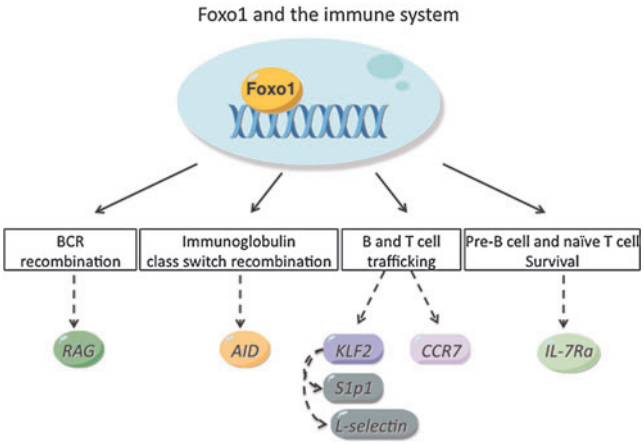

FIG. 6. Foxo1 and the immune system. B and T lymphocytes development, trafficking, and survival are under the transcirptionnal control of Foxo1. Target genes of Foxo1 are indicated in italics. AID, activation-induced (Cytidine) deaminase; RAG, recombination activation gene. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

hinder an unequivocal interpretation of these results. So the precise role of Foxo1 in T cell tolerance and the mechanisms involved still remains to be investigated.

Strikingly, in B cells, Foxo1 has also been identified as a critical inductor of the recombination activation genes Rag1 and Rag2 expression. *In vitro* assays using reporter-based cell systems first showed that overexpression of a constitutively active mutant of Foxo3 induces Rag expression (37). This was further correlated with a decreased proportion of pre-B cells in the bone marrow of *Foxo3*$^{-/-}$ mice, despite normal B cell populations in periphery (37, 39). However, concurrent studies using an *in vitro* gene-reporter model showed that Gadd45-mediated induction of *Rag1,2* expression was specifically inhibited when Foxo1, but not Foxo3 or Foxo4, was knocked down (2). This was confirmed *in vivo*, as deletion of Foxo1 in mice at early stages of B cell development results in a block at the pre-B cell stage, impaired Ig genes rearrangements, and reduced Rag1 and Rag2 expression (19). Although Foxo1 binds directly into the Erag enhancer, which regulates Rag expression during B cell development, Foxo1 is still able to induce Rag expression in Erag-deficient mouse B cells (2, 19). Thus, the precise molecular mechanisms underlying Foxo1-dependent control of Rag expression still need to be clarified. Importantly, Foxo3$^{-/-}$ pre-B cells express normal levels of *Rag2* mRNA and no defects in the rearrangement or assembly of Ig genes were noted in *Foxo3*$^{-/-}$ mice. Further, when *Foxo1*$^{f/f}$ Cd19Cre and *Foxo3*$^{-/-}$ alleles were crossed, no additional phenotypic alterations were noticed. Foxo1 therefore exerts a nonredundant role in Rag expression (19, 39). Moreover, this function of Foxo1 is B cell specific as early deletion of Foxo1 in the T cell lineage does not affect T cell development, and Foxo1 overexpression thymocytes cell lines does not lead to Rag expression (2, 47).

As described above, *in vitro* experiments suggested an antiproliferative and pro-apoptotic role for Foxo1 in B cells. Surprisingly, the use of mice in which *Foxo1* is specifically deleted in mature B cells has highlighted another role for Foxo1. Immunization of Foxo1$^{f/f}$ Cd21Cre with NP-KLH, a T cell-dependent antigen, induced a robust activation of B cells as assessed by germinal center formation and IgM secretion (19). However, Foxo1-deficient mature B cells failed to undergo class-switch recombination with the resulting deficiency in IgG production (19). Consistent with these results, Foxo1-deficient mature B cells express very low levels of the activation-induced (Cytidine) deaminase (AID) (*Aicda*), the master regulator of the germinal center reaction, including class-switch recombination and somatic hypermutation (19). As for Rag1,2 expression, this effect seems to rely on nonredundant Foxo1 functions as activated Foxo3$^{-/-}$ B cells express normal levels of AID. Whether Foxo1 directly regulates expression of AID is unknown; however, once again this reveals a nonredundant and highly specialized function for Foxo1—this time in the biology of B cells.

Strikingly, so far all these recent results have highlighted a predominant role of Foxo1 in B and T lymphocytes biology as opposed to Foxo3. Further, contrary to what was expected from a Foxo loss of function, every analysis of mice with selective Foxo1 deficiencies in T and/or B cells failed to reveal increased resistance to apoptosis or uncontrolled proliferation. No defects in Bim expression have been reported in either T or B cells (19, 47). Rather, these studies identified a critical role for Foxo1 in completely unanticipated immune system-associated functions such as trafficking, gene rearrangements, and lymphocyte survival (Fig. 7). Interestingly, most, if not all, of these processes driven by Foxo1 are associated with the activation of the PI3K-AKT pathway: IL-7 signaling; expression of the pre-BCR and the BCR during B cell development; and the germinal center reaction (Fig. 8). Consequently, this places Foxo1 in a central position regarding negative feedback regulatory loops involved in B and T cell development, homeostasis, and tolerance. By extension, Foxo1 acts as a critical integrator in multiple critical checkpoints that maintain the integrity of the adaptive immune system.

Foxo Redundancy

Despite differences in their relative expression levels, expression patterns of the different Foxo members reveal extensive overlap. Regarding the mouse and human immune system, biochemical and genetic studies clearly indicate

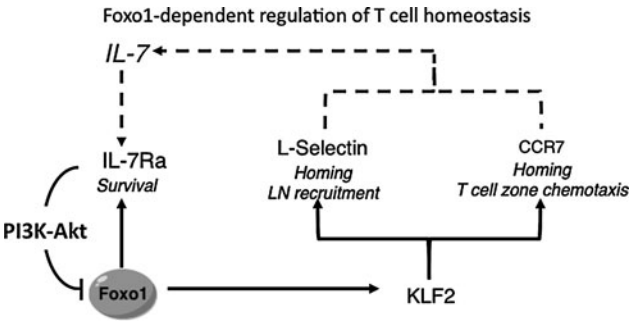

FIG. 7. Foxo1-dependent regulation of T cell homeostasis. Foxo1 links homing and survival of naive T cells by regulating L-selectin, CCR7 and IL-7Ra. IL-7Ra, IL-7 receptor a.

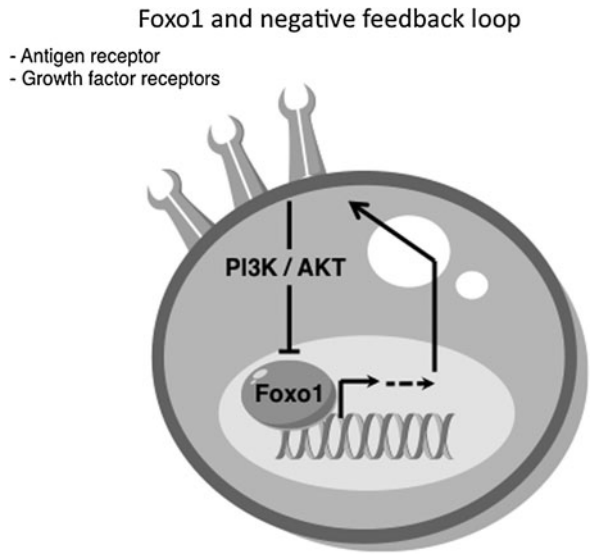

FIG. 8. Foxo1 and negative feedback loop. Foxo1 is essential in the negative-feedback control of growth factor receptors expression such as IL7Ra or the insulin receptor. In the presence of growth factors, the PI3K/Akt-dependent Foxo1 inhibition will lead to the downregulation of growth factor receptor expression.

that many cell populations express at least two or three Foxo members. Experiments that examined the sequence binding specificity of Foxo paralogs, Foxo1, Foxo3, and Foxo4, showed that they all recognize the same DNA binding motif 5′-GTAAACAA-3′ (7, 29). As discussed above, biochemical studies *in vitro* or *ex vivo* have shown that Foxo1, Foxo3, and Foxo4 are targeted by many of the same posttranslational-modifying enzymes, largely behave similarly regarding cytoplasmic/nuclear shuttling, and appear to be able to regulate many common target genes involved in cell cycle control and apoptosis. Consistent with this, sequence alignments indicate that the regions with the highest sequence identity include the N-terminal region surrounding the first AKT phosphorylation site, the Forkhead DNA-binding domain, the region containing the nuclear localization signal, and a part of the C-terminal transactivation domain (60). *In vivo*, only simultaneous deletion of all six copies of Foxo1, 3, and 4 engender cancer-prone conditions with progressive development of thymomas and hemangiomas (63). This phenotype was suppressed by even a single Foxo allele. In addition, most *in vitro* studies using Foxo overexpression systems demonstrated that individual Foxo members regulate common target genes. The transcription programs of Foxo factors have thus long been thought to be highly redundant.

Yet, genetic studies in mice or cell-based systems, some of them being discussed here in the context of the immune system, support unique functions for Foxo1 or Foxo3. Relative expression or activity of different Foxo transcription factors in a single cell type is difficult to rigorously determine. Thus, it is difficult to unambiguously conclude that the observed effects in single knock-out or knock-down models are really due to specific roles rather than differential expression and/or differences in relative activity. Nonetheless, at least in T and B cells, it is now clear that despite significant expression of Foxo3, the deletion of Foxo1 has drastic consequences *in vivo*, whereas the deletion of Foxo3 thus far has no obvious effects (18, 19, 47). Thus, we hypothesized that specialized functions may be controlled by individual factors or cofactor complexes, whereas genes involved in fundamental cellular functions such as survival, cell cycle progression, and stress resistance may be controlled in a redundant manner by two, three, or even four Foxo factors. Hence, specialized functions can be controlled independently from fundamental functions. This may explain why, contrary to the role of Foxo in cell cycle inhibition and apoptosis, neither enhanced spontaneous proliferation nor enhanced resistance to apoptosis has been noticed in single knock-out animals (19, 47). Therefore, remaining expression of other Foxo's could still compensate to control expression of common target genes *in vivo*. Of note, given the striking differences between results derived from the analysis of mutant mice compared with those from *in vitro* overexpression, the interpretation of experiments that use constitutively active mutants of Foxo1 or Foxo3 could be difficult.

Regarding B cells, the deletion of both Foxo1 and 3 at the level of pre-B cells ($Foxo1^{f/f}$ Cd19Cre mice) did not lead to any obvious additional phenotypic alterations compared with the single deletion of Foxo1 (19). However, relative expression of Foxo4 in the concerned cell types is not known, leaving open the possibility that its additional deletion could lead to B cell lymphomagenesis. To date, several reports indicate that mature T cells express Foxo1 and Foxo3, whereas, to our knowledge, no reports support expression of Foxo4, and real-time (RT) quantitative polymerase chain reaction analyses carried in our lab failed to detect it (Y.M. Kerdiles and S.M. Hedrick, unpublished data). The analysis of mice deficient for both Foxo1 and Foxo3 specifically in T cells could therefore represent an informative model to dissect and study the mechanisms governing the redundant *versus* specific functions of Foxo transcription factors.

Given these considerations, how would specialized *versus* redundant functions of Foxo transcription factors be determined and segregated? No obvious differences in terms of targeting by molecular pathways and/or regulation of their own expression have been described so far. Consequently, one likely hypothesis is that the specific roles for each of the Foxo factors are determined by their selective association with cofactors through their most divergent molecular domains (75). The redundant and more basic Foxo functions such as cell cycle inhibition and apoptosis induction may be preferentially controlled *sans* cofactors or through unspecific interactions. Of note, another level of regulation and specialization playing a role in this phenomenon might stem from differences in their relative expression patterns, as exemplified by the specific and conserved expression of Foxo1 in T and B cells and ovaries, as well as Foxo6 in the brain. In any case, although it is now clear that Foxo transcription factors play a critical role in the immune system, these observations highlight the importance of analyzing the consequences of deleting multiple Foxo members in specific cell types.

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Abbreviations Used

ABL = Abelson (Abl) tyrosine kinase
AID = activation-induced (Cytidine) deaminase
Akt = serine/threonine protein kinase, also known as protein kinase B
BCR = B cell antigen receptor
BMDC = bone marrow-derived dendritic cell
CTLA4 = cytotoxic T-lymphocyte antigen 4
DC = dendritic cell
EC = elite controller
Foxo = Forkhead box O
GILZ = glucocorticoid-induced leucine zipper
HSC = hematopoietic stem cell
ICOS = inducible costimulator
IDO = indoleamine 2,3-dioxygenase
IFN = interferon
Ig = immunoglobulin
IL = interleukin
IL-7Rα = IL-7 receptor α
LCMV = lymphocytic choriomeningitis virus
MCP1 = monocyte chemotactic protein-1
NF-κB = nuclear factor-kappa B
NK cells = natural killer cells
PI3K = phosphatidylinositol 3-kinase
Rag = recombination activation genes
RT = real-time
TCR = T cell antigen receptor
TGF = transforming growth factor
TLRs = toll-like receptors
TNF = tumor necrosis factor
Treg cell = regulatory T cell
WT = wild type