

The AKT–mTOR axis regulates de novo differentiation of CD4⁺Foxp3⁺ cells

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CD4⁺Foxp3⁺ regulatory T (T reg) cells play an essential role in maintaining immunological tolerance via their suppressive function on conventional CD4⁺ T (T_{conv}) cells. Repertoire studies suggest that distinct T cell receptor signaling pathways lead to T reg differentiation, but the signals that regulate T reg specification are largely unknown. We identify AKT as a strong repressor of entry into the T reg phenotype in vitro and in vivo. A constitutively active allele of AKT substantially diminished TGF- β -induced Foxp3 expression in a kinase-dependent manner and via a rapamycin-sensitive pathway, implicating the AKT–mammalian target of rapamycin axis. The observed impairment in Foxp3 induction was part of a broad dampening of the typical T reg transcriptional signature. Expression of active AKT at a stage before Foxp3 turn on during normal T reg differentiation in the thymus selectively impaired differentiation of CD4⁺Foxp3⁺ cells without any alteration in the positive selection of T_{conv}. Activated AKT, in contrast, did not affect established Foxp3 expression in T reg cells. These results place AKT at a nexus of signaling pathways whose proper activation has a strong and broad impact on the onset of T reg specification.

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Abbreviations used: AKT*, constitutively active AKT; CTRL, control; DN, double negative; DP, double positive; mTOR, mammalian target of rapamycin; PI3K, phosphoinositide 3-kinase; SP, single positive; T_{conv} cell, conventional CD4⁺ T cell.

Immunological tolerance ensures proper discrimination of self from nonself by the immune system. Despite extensive pruning of differentiating thymocytes to eliminate self-reactive specificities, potentially self-destructive cells do escape into the periphery, necessitating the mobilization of peripheral regulatory mechanisms to prevent autoimmunity (1). One such mechanism is the suppressive action of a particular lineage of T reg cells (2) that expresses the winged-helix/forkhead transcription factor Foxp3, routinely comprising 5–10% of CD4⁺ T cells in normal, unmanipulated individuals (3). The critical role of Foxp3 in T reg cells is evident in the lymphoproliferative disorder and multi-organ autoimmunity accompanying deficiencies in this molecule both in humans and mice (4). Furthermore, ectopic expression of Foxp3 is sufficient, in certain experimental settings, to impart suppressive function to conventional CD4⁺ (T_{conv}) cells (5). However, more recent data have provided a revised view of the sufficiency of and necessity for Foxp3 in the generation and sustenance of the T reg lineage (6–8).

CD4⁺Foxp3⁺ cells differentiate in the thymus and may also be generated in the periphery by antigen-driven conversion of naive T_{conv}

cells under certain conditions (for review see reference 9). In addition, in vitro activation of peripheral CD4⁺CD25⁺ T cells in the presence of TGF- β and IL-2 induces Foxp3 expression and conversion to T reg cells in various model systems (10, 11). More recent evidence indicates that even though TGF- β is not critical for thymic generation of T reg cells, this factor is important for maintenance of the peripheral T reg population (12–15).

Despite an extensive literature on the function of T reg cells in a variety of contexts, little is known about the signaling mechanisms involved in their differentiation and maintenance, or in the regulation of Foxp3 expression. Several papers document defects in signaling molecules downstream of the TCR or of co-stimulatory receptors that affect the differentiation of both the CD4 single-positive (SP) T_{conv} population and the T reg lineage (references 16–19; for review see reference 20). On the other hand, the identification of signaling factors specifically involved in T reg cell differentiation has remained elusive. Differentiation of thymic T reg cells, like that of conventional CD4SP thymocytes, is highly dependent on signals emanating from the TCR (3, 20). The repertoire of TCRs displayed by T reg cells is distinct from that of T_{conv} cells (21–24) and appears to be

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enriched for self-reactive specificities (22, 25). It has been proposed that T reg cell differentiation relies on TCRs with high affinity for MHC–self-peptide complexes (26–28), although this preference is not necessarily imparted by all high-affinity TCR interactions (29–31). Thus, although common signaling pathways coordinate the differentiation of Tconv and T reg cells, it is not clear whether quantitative or qualitative differences account for differentiation into the two lineages.

The phenotypic and functional differences between Tconv and T reg cells are accompanied by subtle differences in signaling pathways. It has been reported that mature peripheral T reg cells, both in humans and mice, display a distinct signaling pattern downstream of the TCR (32) and the IL-2R (33) when compared with Tconv cells. Most notably, the activation of the phosphoinositide 3-kinase (PI3K)–AKT pathway, as indicated by a reduction in AKT phosphorylation, was impaired in T reg cells. However, it remains unclear what the significance of this impairment might be in regards to T reg differentiation and maintenance. The PI3K–AKT pathway is often deregulated in immune disease, and expression of a constitutively active AKT (AKT*) leads to autoimmunity (34, 35). In this paper, we address the functional consequence of deregulated AKT activity on T reg differentiation and Foxp3 expression. Our results describe a unique and surprising effect of AKT in negatively regulating the induction of a broad swath of the T reg transcriptional signature, thereby identifying a signaling component that selectively affects T reg lineage differentiation.

RESULTS

AKT* impairs de novo Foxp3 induction by TGF- β

To explore the functional relevance of a hyperactivated PI3K–AKT pathway on T reg cell generation and maintenance, we first used an *in vitro* system wherein the activation of CD4 $^+$ CD25 $^-$ cells in the presence of TGF- β and IL-2 induces de novo expression of Foxp3 (10). This *in vitro* system is amenable to retroviral gene transfer, facilitating experiments aimed at addressing the influence of diverse molecules on Foxp3 induction. Purified CD4 $^+$ CD25 $^-$ cells were activated with anti-CD3/CD28 beads in the presence of TGF- β and IL-2 for 24 h, at which time they were retrovirally transduced to express AKT* (a myristoylated allele of AKT), which allows its constitutive association with the plasma membrane and thereby its constitutive activation (36). In addition to AKT*, the retroviral vectors encoded either a GFP or Thy1.1 marker, which allowed the discrimination between infected and noninfected cells in the same cultures, the latter serving as internal controls (CTRLs). After 72 h, Foxp3 expression was quantitated by flow cytometry in the infected (GFP $^+$) and noninfected (GFP $^-$) populations. Retrovirus-mediated expression of AKT* significantly impaired the induction of Foxp3 by TGF- β (Fig. 1 A; a summary of several independent experiments is shown in Fig. 1 B). This effect was specific for and intrinsic to the AKT*-expressing cells, with no effect on GFP $^-$ uninfected cells in the same cultures. The diminished expression of Foxp3 at the protein level was

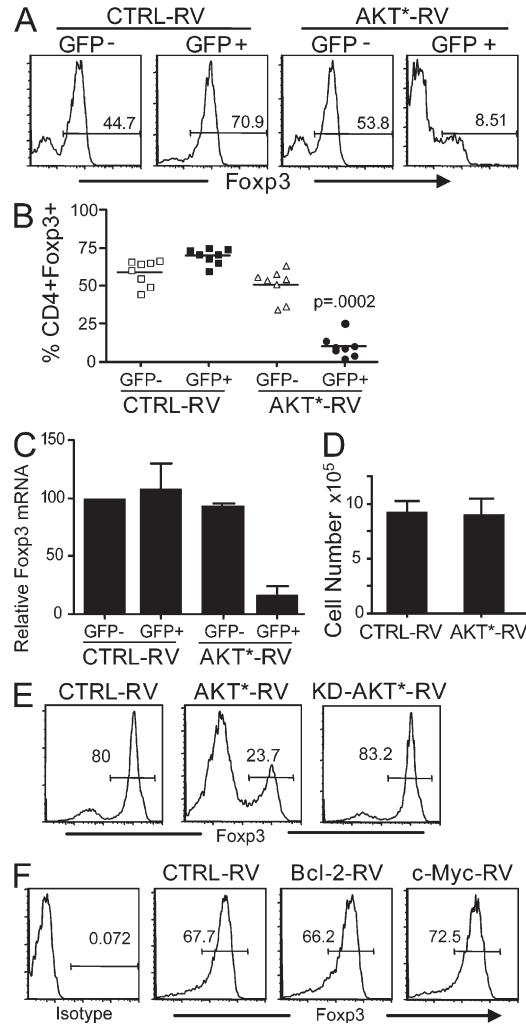


Figure 1. AKT* impairs de novo induction of Foxp3 by TGF- β . Naive CD4 $^+$ CD25 $^-$ T cells were stimulated with anti-CD3/CD28 beads in the presence of TGF- β and IL-2. 24 h after activation, cells were transduced with retrovirus encoding either empty vector control (CTRL-RV) or a constitutively active form of AKT (AKT*-RV; A). After a total of 4 d in culture, cells were analyzed for intracellular levels of Foxp3 by flow cytometry. The percentages of cells positive for Foxp3 are shown. (B) Summary of eight independent experiments quantifying Foxp3 expression by flow cytometry analysis on the indicated populations. Horizontal lines indicate the mean value for each sample. (C) Real-time PCR analysis for Foxp3 mRNA levels in the indicated populations. Foxp3 mRNA is normalized to HPRT mRNA. Data represent the mean \pm SEM for three independent experiments. (D) The experiment was performed as in A, and cell numbers were determined at the end of culture. (E) Naive CD4 $^+$ CD25 $^-$ T cells were stimulated in the presence of TGF- β and IL-2. 24 h after activation, cells were transduced with retrovirus expressing AKT* or a kinase dead (KD) AKT. Expression of Foxp3 was determined after a total of 4 d of culture. The percentages of cells positive for Foxp3 are shown. (F) Similar to the experiment in A, except that T cells were transduced with retrovirus expressing Bcl-2 or c-myc.

paralleled at the mRNA level, pointing to an effect on mRNA transcription or stability (Fig. 1 C). Furthermore, the observed impairment was not caused by a perturbation of the overall activation of the cells: similar cell numbers were observed at

the end of the culture in the presence or absence of AKT* (Fig. 1 D). As expected, our AKT* vector did promote cell survival in conditions of cytokine deprivation (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20071477/DC1>). The ability of AKT* to block Foxp3 induction was dependent on its kinase activity; a mutant AKT that shares the myristoylated modification found in AKT* but carries an inactivating mutation of the kinase domain (KD-AKT(K179M)) (37) was unable to affect TGF- β -mediated Foxp3 induction (Fig. 1 E). To the extent tested, the effect of AKT* on Foxp3 induction was independent of genetic background, because both NOD and B6 mice showed a similar change of downmodulation (unpublished data). This effect of AKT* was specific, in that no significant difference in Foxp3 induction was observed upon expression of the antiapoptotic molecule Bcl-2 or the protooncogene c-Myc, which, as with AKT, have been implicated in T cell survival (Fig. 1 F).

To explore the time window during which AKT* could exert its inhibitory effect on Foxp3 induction, we transduced cells with AKT* at various time points after activation, always performing the cytofluorimetric analysis 3 d later. The inhibitory effect was most apparent when AKT* was introduced at early time points after activation (Fig. 2 A, top), evident as both fewer Foxp3⁺ cells and a reduction in staining intensity in those cells expressing Foxp3. The reduction in the number of Foxp3⁺ cells was less severe at later time points, although AKT* could still reduce the intensity of Foxp3 expression in positive cells (Fig. 2 A, bottom). The continuous presence of TGF- β is necessary to maintain Foxp3 expression in such cultures; i.e., Foxp3 begins to disappear within 24 h of TGF- β withdrawal (unpublished data) (38). AKT* seems to affect this continued “confirmation” of Foxp3 expression as well as the initial induction.

To confirm this notion, we tested the effect of AKT* overexpression on Foxp3 levels in primary T reg cells, whose expression of Foxp3 is much more stable in culture (8). T reg cells were purified as CD4⁺CD25^{hi}CD62L^{hi} and were activated ex vivo in the presence of TGF- β and IL-2 to allow cell-cycle entry and, thus, infectivity by retroviruses. Contrary to what we had observed for the TGF- β -induced Foxp3 in Tconv cells, the Foxp3 levels in the natural T reg cell cultures were refractory to downmodulation upon retroviral expression of AKT* (Fig. 2 B). Although transduced T reg cells did tend to express slightly lower levels of AKT* overall (as suggested by the bicistronic Thy1.1 reporter), this resistance was not caused by a lower level of AKT* because the resistance was observed at AKT*/Thy1.1 levels that elicited complete inhibition in TGF- β -treated naive cells (Fig. 2 B). Thus, there appear to be differences in the molecular circuitry regulating Foxp3 induction or maintenance in these two populations, as reflected by their sensitivity to AKT*.

Broad but partial impairment of the T reg transcriptional signature by AKT*

These findings raised the question of whether the observed inhibition of Foxp3 induction was a consequence of an overall

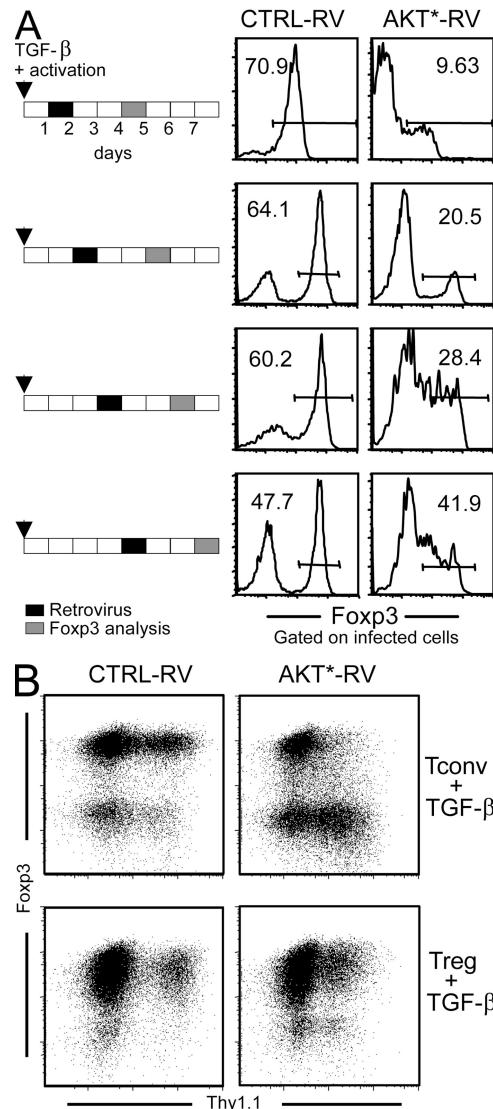


Figure 2. Differential effect of AKT on established levels of Foxp3. (A) Naive CD4⁺CD25⁻ T cells were activated as before and transduced with retrovirus at the indicated time points. Foxp3 expression was determined by flow cytometry analysis. The percentages of cells positive for Foxp3 are shown. (B) Naive CD4⁺CD25⁻ T cells or CD4⁺CD25^{hi} T reg cells were stimulated in the presence of TGF- β . T reg cell stimulation was done in the presence of 2,000 U IL-2. Cells were transduced with retrovirus 72 h after activation and analyzed for Foxp3 by flow cytometry after an additional 72 h of culture. Data are representative of three or more experiments.

blockade of TGF- β signaling or a specific inhibition of Foxp3, or whether Foxp3 typified a gene set targeted by AKT*. To address this question, we examined the transcript profiles of conventional CD4⁺ T cells cultured in TGF- β and infected by either a retrovirus encoding AKT* or a CTRL retrovirus. Total RNA from purified cell populations was purified, amplified, and hybridized to M430 2.0 Affymetrix whole-genome mouse arrays. Data were normalized using the rate monotonic algorithm and were averaged between

three independent replicates. The Multiplot module from GenePattern (39) was used for data visualization.

Overall, the introduction of AKT* led to a substantial number of changes in the overall profile of TCR/IL-2/TGF- β -activated cells: at an arbitrary fold change threshold of 1.5, 610 and 1,381 probes were up- and down-regulated by AKT*, respectively, far more than in the randomized CTRL dataset (Fig. 3 A). Next, we ascertained more specifically the impact AKT* had on the transcript changes imparted by TGF- β (the “TGF- β signature”), thereby testing whether the effect on Foxp3 was isolated or corresponded to a shutdown of the entire TGF- β signature. We compared the effect of TGF- β (as the ratio of expression values in cultures with vs. without TGF- β) in cells that were transduced with AKT* or with the CTRL retrovirus. This comparison is displayed in the fold change/fold change plot of Fig. 3 B, which provides a direct comparison of the TGF- β signature in the presence (y axis) or absence (x axis) of AKT*. The TGF- β signature partitioned

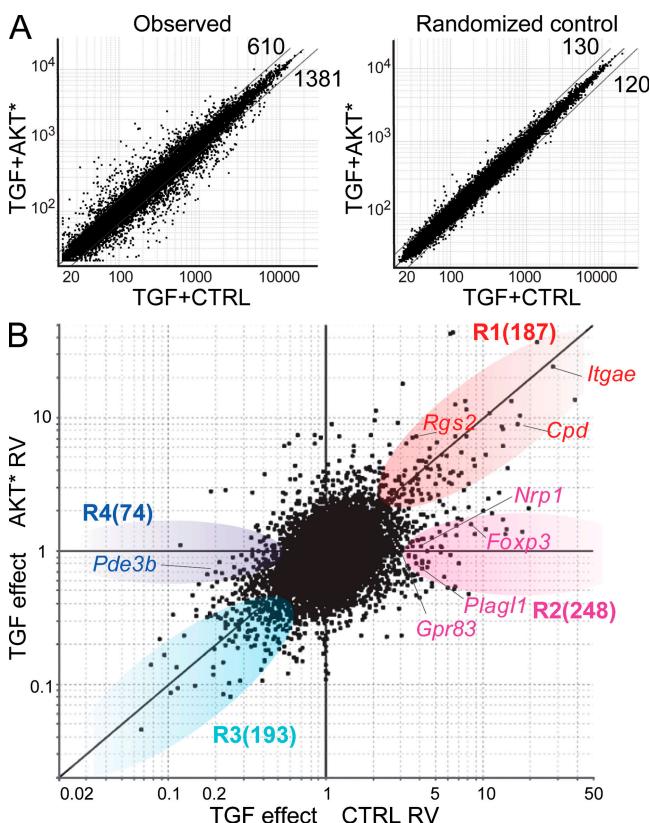


Figure 3. AKT* specifically targets a subset of the TGF- β signature. (A) Comparison of the expression values of TGF- β -activated cells transduced with CTRL or AKT* retrovirus (left) compared with a randomized dataset (right). (B) Fold change versus fold change (FcFc) plot comparing the TGF- β -induced signature in AKT*- versus CTRL-transduced cells. Gene expression values for cells activated in the absence of TGF- β and transduced with a CTRL retrovirus were used as the denominator for the Fc. Regions R1 and R3 highlight TGF- β -regulated genes that are refractory to AKT* expression. Regions R2 and R4 highlight TGF- β -regulated genes that are affected by AKT* expression.

into two distinct clusters of genes differing in their responsiveness to AKT*. The genes along the diagonal line (regions R1 and R3) were equally responsive to TGF- β regardless of the presence or absence of AKT*. This group included *Itgae* (encoding CD103), *Rgs2*, and *Cpd*. In contrast, for those genes that fell within regions R2 or R4, the change in their expression promoted by TGF- β was reduced or abolished by AKT*. As expected, *Foxp3* belonged to this group, which also included canonical T reg transcripts such as *Plagl1* or *Nrp1*. Overall, AKT* shut down or strongly curtailed \sim 30% of the transcripts induced by TGF- β and \sim 20% of TGF- β -repressed transcripts. Thus, AKT* did not elicit a wholesale block of TGF- β signaling nor did it solely shut down *Foxp3*, but rather, it targeted a specific subset of genes, one of which was *Foxp3*.

Because AKT* affected several genes other than *Foxp3*, we asked what its overall impact was on the transcriptional signature that defines T reg cells. We have robustly defined elsewhere a consensus “T reg signature” deduced from the juxtaposition of several T reg and Tconv datasets (8). In Fig. 4, the changes elicited by AKT* in these T reg signature transcripts are plotted in a volcano plot representation (fold change vs. p-value; genes over- and underexpressed in T reg cells are shown in red and blue, respectively). It is clear that AKT* affected a substantial portion of the T reg signature: of 407 genes overexpressed in T reg cells, 292 were dampened by AKT*, whereas 125 out of 196 of the underexpressed genes were actually boosted by AKT* in the TGF- β -induced cultures ($P = 4 \times 10^{-38}$ and 8.2×10^{-5} for the up- and down-regulated probes, respectively, according to the χ^2 test). Notably, the induction of several canonical T reg genes, such as *Il2ra*, *Ctla-4*, *Tnfrsf18*, and *Nrp1*, in addition to *Foxp3* itself, was reduced by AKT*. Conversely, AKT* increased the expression of several genes normally repressed in T reg cells, such as *Pde3b* (6). Thus, activated AKT dampens a broad swath of the genes whose differential expression characterizes T reg cells.

A sizeable component of the T reg signature corresponds to genes that respond to IL-2 and T cell activation (8), and IL-2 is an essential growth/survival factor for T reg cells (40), raising the possibility that the effect of AKT* might be to dampen IL-2 and/or activation signals. To this end, we analyzed the response of a set of IL-2/activation signature genes in these cultures (Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20071477/DC1>): although AKT* did affect a substantial proportion of these transcripts, the effect was incomplete, indicating that it does not uniformly dampen IL-2 signaling.

These observations raised the question of whether the other affected genes were directly influenced by AKT* or were downstream targets of *Foxp3* and, as such, mirrored its downmodulation. To address this issue, we made use of existing microarray datasets from cells transduced with *Foxp3* or CTRL retrovirus, which thus define a “*Foxp3* signature” (i.e., the group of genes directly influenced by *Foxp3*) (8). Recently, we have shown that the transcriptional profile imparted by *Foxp3* represents only a subset of the entire T reg signature (8). Highlighting those genes that were up-regulated

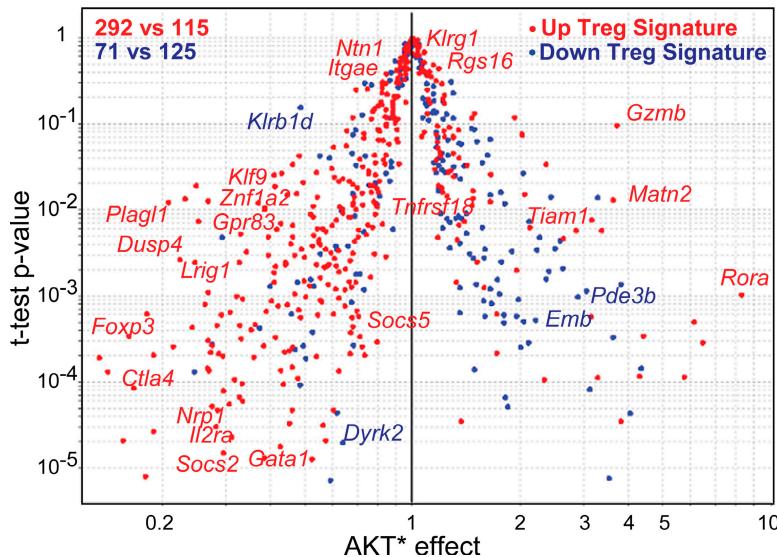


Figure 4. AKT* significantly impairs a subset of T reg signature. Volcano plot representation (fold change vs. p-value) of the effect of AKT* expression (ratio of TGF-AKT* to TGF-GFP) on a common T reg signature (red, up; blue, down). Numbers indicate the probes present in either of the regions.

(in red) or down-regulated (in blue) by Foxp3 transduction (Fig. 5 A) showed that the effect of AKT* could not simply be ascribed to the lowering of Foxp3: although some Foxp3-responsive genes did belong to region R2/R4 of Fig. 3 B (their downmodulation mirroring that of Foxp3), this was not the case for all Foxp3-responsive genes. In addition, many genes not responsive to Foxp3 (gray) were part of region R2 as well. This distribution argued against a model in which the observed downmodulation of R2 genes by AKT* was simply the result of a shutdown of Foxp3 expression. For an independent confirmation of this result, we performed dual-transduction experiments in which Foxp3 levels in AKT*-transduced cells were restored by coinfection with a retroviral vector encoding FoxP3 (Fig. 5 B). This protocol allowed us to compare mRNA levels for target genes in cells expressing AKT* with or without additional FoxP3 (Fig. 5 C). The forced expression of exogenous Foxp3 restored the levels of Foxp3 transcripts comparable to those of uninfected cells (Fig. 5 C). However, restoration of Foxp3 did not restore the expression levels of *Ctla4* or *Gpr83* transcripts (Fig. 5 C), validating the conclusion that AKT* affected transcripts of the T reg signature independent of its effect on Foxp3.

Effect of AKT on de novo generation of Foxp3⁺CD4⁺ thymocytes

T reg cells are viewed as a distinct lineage that arises in the thymus and depends on interactions between the TCR and MHC-peptide complexes (20). Given the broad effect of AKT* on the T reg signature in mature T cells activated in the presence of TGF- β , we explored AKT's impact on the commitment of differentiating thymocytes to the T reg lineage in a more physiological setting. To this end, it was first necessary to establish an experimental system into which we could introduce AKT* at a stage before Foxp3 turns on. Foxp3 expression

can be observed as early as the CD4⁺CD8⁺ double-positive (DP) stage of thymocyte differentiation; T reg cells accumulate as maturing cells (41, 42). Thus, we chose to infect the immediate precursors of DP cells, double-negative (DN) thymocytes, and to reimplant these directly into the thymus of recipient mice to follow their natural maturation (Fig. 6 A). DN thymocytes harvested from congenically marked CD45.1⁺ mice were transduced with a CTRL retrovirus or with one expressing AKT*. Both vectors also encode the Thy1.1 marker for the detection of transduced cells. The transduced cells were injected intrathymically into irradiated CD45.2⁺ mice, leading to a wave of differentiation in the following 10–20 d. This system allows differentiation to occur in the proper thymic milieu and avoids issues that can occur upon expression of the transgene at earlier stages, e.g., in bone marrow stem cells. 14 d after injection, we examined the ability of the AKT*-transduced thymocytes to differentiate into CD4⁺Foxp3⁺ cells. Although uninfected (Thy1.1⁻) cells or Thy1.1⁺ cells infected with the CTRL vector gave rise to normal proportions of Foxp3⁺ cells among CD4SPs, there was a striking reduction in the differentiation of Foxp3⁺ cells from the AKT*-transduced DN precursors (Fig. 6 B; several experiments are compiled in Fig. 6 C). On the other hand AKT*, expression did not significantly alter the differentiation of conventional CD4SP populations, as determined from (a) the CD4/8 profiles of Thy1.1⁺ cells (Fig. 6 D), (b) the ratio of positively selected Tconv cells relative to DPs (note that there is quite some variability between mice for this metric, which varies with the continued differentiation potential of the donor DNs, but that the distribution is similar for cells transduced with AKT* and CTRL vectors; Fig. 6 E), and (c) the ratio of positively selected CD4- and CD8SPs (Fig. 6 F). These results highlight AKT's selective impact on the differentiation of CD4⁺Foxp3⁺ T reg cells, with no overt

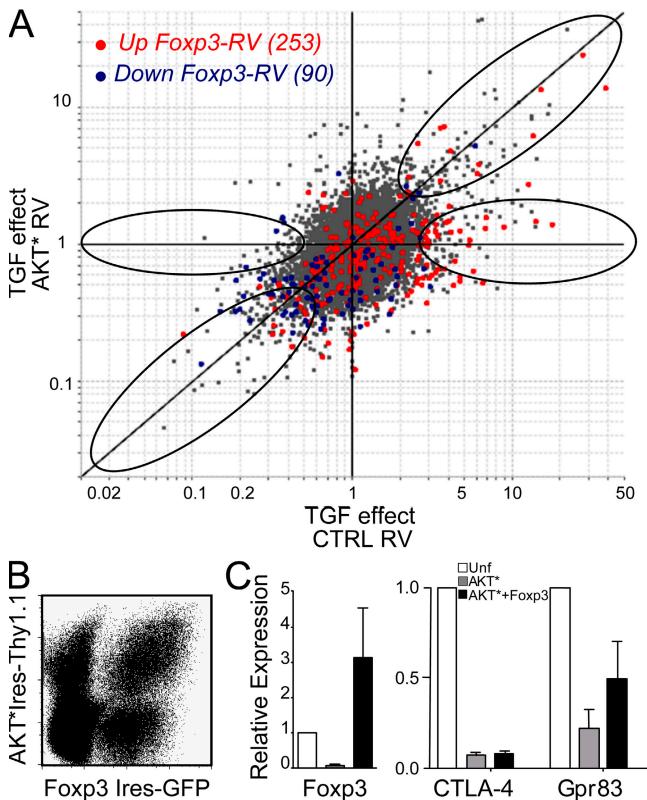


Figure 5. Impact of AKT* on Foxp3-dependent and -independent genes. (A) Similar to the experiment in Fig. 3 B, except that genes up-regulated (red) or down-regulated (blue) by Foxp3 retroviral transduction are highlighted. Regions R1, R2, R3, and R4 are outlined. Naive CD4⁺CD25⁻ T cells were activated as before and transduced with the indicated retrovirus. (B) Naive CD4⁺ T cells were activated in the presence with CD3/CD28 beads in the presence of TGF/IL-2 and transduced with AKT*-Thy1.1 and Foxp3-GFP retrovirus. (C) Real-time PCR analysis for Foxp3, CTLA-4, and Gpr83 mRNA was performed. Data represent the mean \pm SEM.

effect on Tconv differentiation. We are not aware of any other element with this property.

Signaling from AKT to inhibit Foxp3 induction

As we showed in Fig. 1 E, the ability of AKT* to block Foxp3 induction was dependent on its kinase activity. To further explore the signaling pathways involved in T reg differentiation, we sought to determine whether this effect was mediated via the mammalian target of rapamycin (mTOR) pathway. mTOR is a downstream target in the AKT signaling pathway, and recent papers have proposed a link between rapamycin and T reg cells both in humans and mice, with rapamycin preferentially sparing T reg cells (43–46). We explored mTOR's involvement in AKT*'s down-modulatory activity by adding rapamycin to cultures of CD3/IL-2/TGF- β -triggered CD4⁺ cells transduced either by AKT* or CTRL retrovirus. By itself, rapamycin did not affect TGF- β -mediated induction of Foxp3 (Fig. 7). However, rapamycin treatment of cells at the time of AKT* transduction did counteract the

inhibitory effect of AKT*, although it did not completely restore Foxp3 levels to those of uninhibited cells. These results identify mTOR and, more specifically, the rapamycin-sensitive branch of mTOR complexes as downstream targets of AKT* in exerting its effect on Foxp3 induction. On the other hand, because the impact of AKT* was not completely abolished by rapamycin, additional rapamycin-insensitive pathways may also be involved.

DISCUSSION

Given the critical role of T reg cells in immune tolerance, it is important to delineate the signaling pathways that positively or negatively regulate their differentiation. In this study, we investigated the functional consequence that deregulated activity of AKT has on Foxp3 induction and T reg specification, and identified AKT as a strong repressor of this process. The ability of AKT to inhibit Foxp3 expression was limited to the induction phase of Foxp3 both in vivo and in vitro. Natural T reg cells with established and stable Foxp3 levels were resistant to AKT*'s effect. This differential sensitivity to AKT* in natural T reg cells or Foxp3 high-expressing cells and the early induced Foxp3⁺ cells underscores the significant differences between these states (8) and suggests distinct chromatin states as one possibility: a “locked-in” state of chromatin at the Foxp3 locus in T reg cells versus a “reversible” one in the early time points of expression, whether resulting from natural commitment to the lineage or from TGF- β induction. Support for this notion was recently provided by Floess et al., who noted complete demethylation of CpG motifs and histone modifications within the Foxp3 locus in natural T reg cells but not after TGF- β induction (47).

The observed impact of AKT* was not attributed to a global transcriptional block downstream of TGF- β signaling nor to a shutdown of IL2 signaling, but rather it was a specific effect on a set of genes that included *Foxp3* and other members of the T reg signature, both *Foxp3* responsive and *Foxp3* nonresponsive. AKT* influenced the majority of the T reg signature genes, although not all of them, such that one can readily envision that T reg commitment would be blocked. These findings lend credence to our current view of the shaping of the T reg transcriptional landscape as a complex interplay of multiple players, with *Foxp3* being an important, but not the sole, factor (6–8, 48). They also implicate AKT in molding the T reg signature and provide an important lead as to how commitment to the alternative T reg and Tconv lineages of CD4⁺ cells can occur in the thymus, resulting in the different TCR repertoires (21–24): TCRs whose interaction with MHC-self-peptide complexes result in a balance of downstream signals with low AKT activation, relative to other signaling pathways, would be those that preferentially enter the T reg lineage. In addition, other signaling components affecting AKT, such as cytokines, might further modulate these signals, perhaps in favored thymic niches. Such differential involvement of signaling molecules downstream of the TCR and/or CD28 in two different fates is reminiscent of the signaling pathways involved in CD4⁺ versus CD8⁺

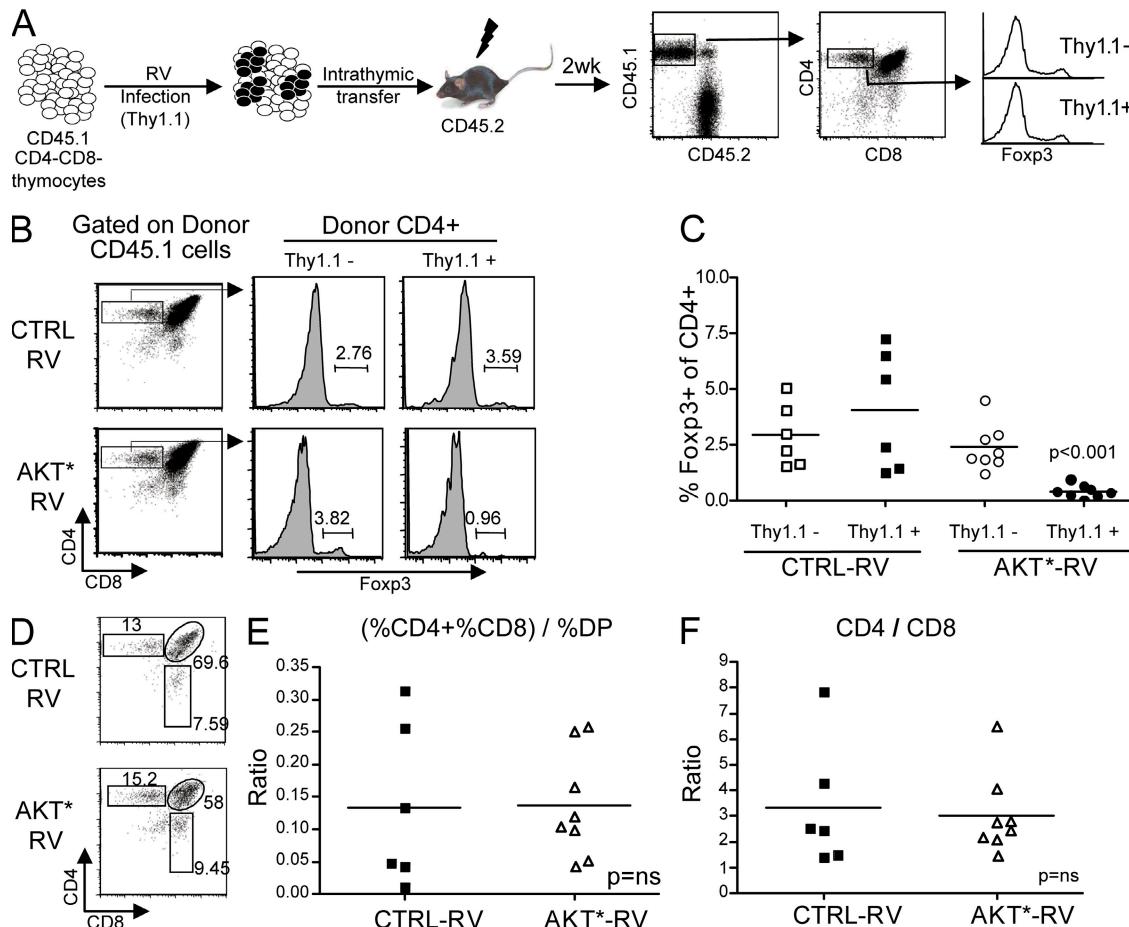


Figure 6. AKT* impairs differentiation of CD4⁺Foxp3⁺ T cells in vivo. (A) Schematic of the intrathymic transfer experiments. CD4⁻CD8⁻ DN thymocytes from CD45.1 mice were transduced with retrovirus encoding AKT* or CTRL vector and injected into sublethally irradiated CD45.2 B6 mice. 2 wk after transfer, donor CD45.1⁺ cells were analyzed for their ability to give rise to CD4⁺Foxp3⁺ cells. (B) Flow cytometry profile of the thymocyte populations gated on donor CD45.1⁺ cells transduced either with CTRL or AKT* retrovirus. Intracellular staining for Foxp3 was performed on electronically gated CD45.1⁺CD4⁺Thy1.1^{-/+} populations. The percentages of cells positive for Foxp3 are shown. (C) Summary of independent experiments ($n = 6$ –8 mice) for the percentage of CD4⁺Foxp3⁺ cells gated on donor cells. Horizontal lines indicate the mean. (D) CD4/CD8 profile of infected (identified by a Thy1.1 retrovirally encoded marker) donor cells. The percentages of cells in the indicated regions are shown. Summary of the ratio of (E) the sum of SP to DP cells and (F) of the CD4SP to CD8SP infected cells from various mice. Horizontal lines indicate the mean.

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T cell lineage commitment or positive and negative selection, whereby fine differences in signal strength can account for opposing outcomes (49–51). These findings would predict a negative impact of CD28 triggering on Foxp3 induction, because PI3K is one of the major signaling paths downstream of CD28. Indeed, such an effect has been reported, where inclusion of anti-CD28 damped the induction of FoxP3 by graded doses of anti-CD3 in a conversion system *in vitro* (52, 53).

Despite contextual differences between the natural differentiation of thymic T reg cells and the induction of Foxp3 in mature T cells by TGF- β , AKT* exerted a similar blocking effect in these two populations. These findings argue for a level of similarity for these two populations and place AKT at a nexus of signaling pathways whose proper activation has a considerable impact on Foxp3 expression and, consequently, T reg cell generation. AKT had previously been implicated

in the growth and survival of CD4⁺ T lymphocytes but not in their differentiation (54). Indeed, given the involvement of AKT as a positive regulator in CD28 and IL-2R signaling (55), both of which are important for Foxp3 expression, our findings may appear somewhat paradoxical.

Activation of AKT is lower in T reg than in Tconv cells after TCR (32) and IL-2R (33) engagement. Our study suggests that this impairment may not merely be a consequence of defective signaling, but that it may serve as an active safeguard mechanism to ensure sufficient levels of Foxp3 and other elements of the T reg signature required for T reg differentiation and for the suppressive functions of T reg cells. An overly active AKT might override these functions and ultimately contribute to immune disease. Indeed, mice expressing AKT* present with autoimmune manifestations such as multiorgan lymphocytic infiltration and autoantibodies (34, 35), although no obvious phenotype in CD4⁺CD25⁺ T cells has

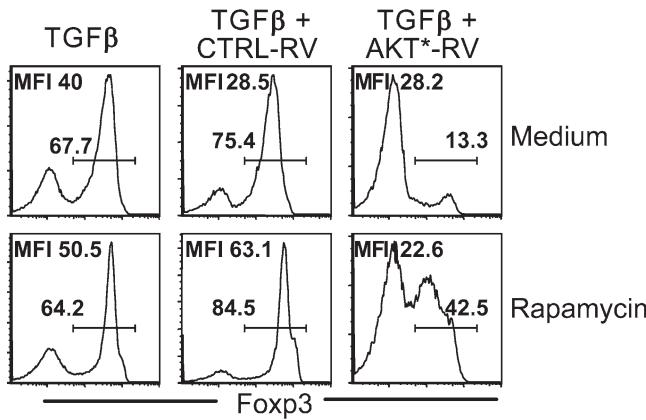


Figure 7. Molecular mechanisms of AKT-mediated suppression of Foxp3 induction. Naive CD4⁺CD25⁻ T cells were stimulated in the presence of TGF- β and IL-2. 24 h after activation, cells were transduced with retrovirus and cultured for an additional 72 h in the presence or absence of 100 nm rapamycin. Foxp3 expression was determined by flow cytometry. The percentages of cells positive for Foxp3 are shown. Data are representative of three or more experiments.

been observed in such mice (56). It has been postulated that autoimmunity in this mouse line could in part be caused by the resistance of effector cells overexpressing AKT to T reg suppression (57). Our study provides additional potential explanations for this phenotype, whereby deregulated expression of AKT alters the thymic and perhaps peripheral generation of T reg cells. An alternative implication may relate to recent data that show that Foxp3 induction by TGF- β can be blocked by proinflammatory cytokines such as IL-6, leading to generation of Th17 cells (58). In light of our findings and given the role of AKT in the IL-6R signaling pathway (59, 60), we propose that IL-6 exerts its inhibitory effect in an AKT-dependent manner. Moreover, these findings suggest that small molecules that target AKT might prove efficacious in treatment of autoimmunity and inflammation by targeting both overreacting effector T cells as well as enhancing T reg cell generation.

The repression of Foxp3 expression by AKT* required its kinase activity and was partly counteracted by rapamycin treatment, adding a potential element to its mechanism of action and placing mTOR as a downstream target of AKT. mTOR belongs to two distinct complexes, mTORC1 and mTORC2, which are differentially sensitive to rapamycin treatment, with mTORC1 being the usual target (61, 62). Given the reversal of AKT*'s effect by rapamycin, our data suggest that AKT is exerting part of its effect via mTORC1. On the other hand, the lack of a complete reversal suggests that mTORC2 may also partially contribute. These results dovetail and provide a possible mechanistic explanation for recent reports documenting a relatively protective effect of rapamycin on T reg cells (43–46). In addition to selectively preserving and expanding existing T reg populations, rapamycin treatment may also contribute to the generation of new T reg cells by counteracting molecular brakes on Foxp3 induction.

MATERIALS AND METHODS

Mice. NOD/LtDOI, BDC2.5/NOD TCR transgenic, C57BL/6, and B6.SJL-Ptprca Pep3b/BoyJ (CD45.1⁺) mice were bred in the mouse specific pathogen-free animal facility at the Joslin Diabetes Center or were purchased from the Jackson Laboratory. All animal experiments approved by the Joslin Diabetes Center Institutional Animal Care and Use Committee.

Cell purification. Naive Tconv cells and T reg cells were harvested from spleens and lymph nodes of 4–6-wk-old BDC2.5/NOD, NOD, or B6 mice. After mechanical disassociation and red blood cell lysis, cells were labeled with anti-CD4 (clone RM4-5) PE–Texas red or PE–Alexa Fluor 610, positively enriched with anti-PE beads (Miltenyi Biotech), and sorted on the following markers: negative for B220 (clone RA3-6B2), CD8 α (clone 5H10), CD11b (clone M1/70), and CD69 (clone H1-2F3; all FITC labeled); positive for CD4; and either positive for CD25 (clone PC61)-allophycocyanin (T reg cells) or negative for CD25 (Tconv cells).

In vitro activation. Naive CD4⁺CD25⁻CD69⁻ T cells were activated with anti-CD3- and anti-CD28-coated beads (Invitrogen) at concentrations of one bead per cell in the presence of 20 U/ml of recombinant human IL-2 (Proleukin; Chiron) with or without 25 ng/ml of recombinant TGF- β (PeproTech). T reg cells were activated with beads and 2,000 U/ml IL-2 in the presence or absence of TGF- β . T cells were cultured for 4 d or longer, as indicated in the figures.

Retroviral transduction. 293FT cells (Invitrogen) were transfected with retroviral expression plasmids (MSCV IRES-Thy1.1/GFP) (63), either empty or encoding the constitutively active allele of AKT (64), human Bcl-2 (63), or human c-myc (65) and the packaging construct pcl-ECO (66) using TransIT-293 (Mirus), according to the manufacturer's instructions. Naive CD4⁺ T cells were activated in the presence of 20 U/ml IL-2 and 25 ng/ml TGF- β and spin infected with retrovirus supernatant at 24 h. Cells were subsequently cultured for an additional 72 h for a total in vitro culture of 4 d. For time-course experiments, cells were analyzed at the times indicated in the figures.

Intrathymic transfer of CD4⁻CD8⁻ T cells. Thymocytes from 3–4-wk-old CD45.1⁺ mice labeled with anti-CD4-PE and anti-CD8-PE–Alexa Fluor 610 were depleted with anti-PE beads (Miltenyi Biotech) using AutoMacs (Miltenyi Biotech). This allowed >90% CD4⁻CD8⁻ population of thymocytes. DN CD4⁻CD8⁻ cells were transduced with retrovirus by spin infection for 1.5 h. After retroviral transduction, ~100,000–200,000 cells in a 10- μ l volume were injected into each thymic lobe of sublethally irradiated (600 rads) C57BL/6 recipients.

Gene expression profiling. Sorted cell populations were lysed in TRIzol reagent, and RNA was prepared according to the manufacturer's instructions (Invitrogen). For retroviral cultures, cells were sorted for GFP/Thy1.1 positivity before RNA processing. RNA amplification was conducted for two rounds using the MessageAmp aRNA kit (Ambion), followed by biotin labeling using the BioArray high yield RNA transcription labeling kit (Enzo Life Sciences, Inc.), and purified using the RNeasy mini kit (QIAGEN). The resulting cRNAs were hybridized to M430 2.0 chips (Affymetrix) by the Joslin Genomics Core, according to manufacturer's instructions. All cell populations used for the microarray analysis were generated in triplicate and individually processed. Raw data were normalized using the rate monotonic algorithm implemented in the Expression File Creator module from the GenePattern software package (39). Probes were filtered based on minimum (>20) and maximum (<20,000) expression values using the Preprocess Dataset module from the GenePattern software. A probe with an expression value <20 was kept if 3 out of the 64 chips showed a value >50. Data were visualized using the Multiplot Preprocess and Multiplot modules from the GenePattern software. Signature depth and correlation coefficient analyses were conducted using S-Plus software (Insightful Corp.). Microarray data have been deposited in the Gene Expression Omnibus under accession no. GSE7596.

Online supplemental material. Fig. S1 shows AKT* promoting T cell survival in the absence of growth factors. Fig. S2 shows the effect of AKT* on IL-2/activation signature. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20071477/DC1>.

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