Cutting Edge

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Cutting Edge: PHLPP Regulates the Development, Function, and Molecular Signaling Pathways of Regulatory T Cells

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Regulatory T cells (Tregs) have a reduced capacity to activate the PI3K/Akt pathway downstream of the TCR, and the resulting low activity of Akt is necessary for their development and function. The molecular basis for the failure of Tregs to activate Akt efficiently, however, remains unknown. We show that PH-domain leucine-rich-repeat protein phosphatase (PHLPP), which dephosphorylates Akt, is upregulated in Tregs, thus suppressing Akt activation. Tregs expressed higher levels of PHLPP than those of conventional T cells, and knockdown of PHLPP1 restored TCR-mediated activation of Akt in Tregs. Consistent with their high Akt activity, the suppressive capacity of Tregs from *PHLPP1*$^{-/-}$ mice was significantly reduced. Moreover, the development of induced Tregs was impaired in *PHLPP1*$^{-/-}$ mice. The increased level of Akt's negative regulator, PHLPP, provides a novel mechanism used by T cells to control the Akt pathway and the first evidence, to our knowledge, for a molecular mechanism underlying the functionally essential reduction of Akt activity in Tregs. *The Journal of Immunology*, **2011, 186: 5533–5537.**

The PI3K pathway is a critical regulator of tolerance, acting as a molecular rheostat for the delivery of signals that enhance cell cycle progression, survival, and proliferation (1). In contrast to conventional T cells (Tconvs), regulatory T cells (Tregs) activated through the TCR or IL-2 receptor fail to stimulate efficiently the PI3K pathway (2, 3). Low PI3K activity in Tregs is required for their suppressive capacity (3), results in Foxo3a- and Foxo1-driven expression of Foxp3 (4–7), and provides the molecular basis for why inhibition of PI3K promotes Treg development and/or function (8, 9). The reason why Tregs fail to stimulate the PI3K pathway is currently unclear.

The serine/threonine phosphatase known as PH-domain leucine-rich repeat protein phosphatase (PHLPP) is a recently identified negative regulator of the PI3K pathway (10). The PHLPP family consists of three isozymes: PHLPP1, which exists as two splice isoforms, α and β, and PHLPP2. All members of this family dephosphorylate the hydrophobic motif of Akt (Ser473 in Akt1) (11). In nonimmune cells, expression of PHLPP blocks Akt activation, triggers apoptosis, suppresses tumor growth, and controls circadian rhythms (11–13).

The TCR-mediated defect in activation of Akt in Tregs is specific for the Ser473 residue in the hydrophobic motif; phosphorylation of Thr308 in the activation loop is normal (3). We speculated that high expression of a Ser473-specific phosphatase such as PHLPP may underlie the inability of Tregs to activate Akt. In this study, we demonstrate that expression of PHLPP is essential for maintaining the paucity of Akt activity in Tregs. Moreover, in the absence of PHLPP, the development and function of Tregs is impaired, demonstrating a previously unknown role for this phosphatase in the regulation of immunological tolerance.

Materials and Methods

Mice and cell isolation

Female C57BL/6 and C57BL/6 Foxp3-EGFP mice (6–12 wk; The Jackson Laboratory) were maintained in specific pathogen-free conditions in accordance with ethics protocols approved by the University of British Columbia Animal Care Committee or the University of California San Diego Institutional Animal Care and Use Committee. CD4$^+$ T cells were sorted into CD4$^+$Foxp3-EGPF^{high} (Treg) and CD4$^+$Foxp3-EGPF$^-$ (Tconv) to >98% purity on a FACSAria. CD4$^+$CD25$^+$ Tregs from *PHLPP1*$^{-/-}$ mice (13) were purified from CD4$^+$ T cells using EasySep CD25 positive selection; Tconvs were CD4$^+$CD25$^-$ (StemCell Technologies).

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Abbreviations used in this article: ChIP, chromatin immunoprecipitation; iTreg, induced regulatory T cell; PHLPP, PH-domain leucine-rich repeat protein phosphatase; siRNA, small interfering RNA; Tconv, conventional T cell; Treg, regulatory T cell; WT, wild-type.

Cell culture and signaling

RPMI 1640 was supplemented with 10% FBS, 10 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate, 1 mM MEM nonessential amino acid solution, and 100 U/ml each of penicillin G and streptomycin. Cells were stimulated with plate-bound CD3 (10 μg/ml; 2C11) and soluble CD28 (1 μg/ml; 37.51). Tregs were differentiated from Tconvs with rhIL-2 (100 U/ml; Chiron) and rhTGF-β (10 ng/ml; R&D Systems). Rapamycin (10 ng/ml; Sigma-Aldrich) and LY294002 (10 μM) were added where indicated. Treg anergy was assessed by culture (5×10^4/well) with or without 50 U/ml rhIL-2. To measure suppression, Tconvs (5×10^4/well) were stimulated with anti-CD3 (0.5 μg/ml), irradiated CD3-depleted splenocytes (1×10^6/well), and serially diluted Tregs. Suppression and phosphorylation of Akt, ERK, or p70-S6 kinase were measured as described (14, 15).

RT-PCR analysis

Gene expression was measured in real time with a sequence detection system (GeneAmp 7300; Applied Biosystems). Primer sequences were as follows: PHLPP1, 5′-GTGCCCTACCTTCTCCAGTG-3′ (forward) and 5′-CACTTGCCAACATTAGCAGA-3′ (reverse); PHLPP2, 5′-CCAGTTGGAACAGGCTGACG-3′ (forward) and 5′-CCAGTGCAGGAAGGACATGG-3′ (reverse); Foxp3, 5′-CCCAGGAAAGACAGCAACCTT-3′ (forward) and 5′-TTCTCACAACCAGGCCACTTG-3′ (reverse); 18S, 5′-CAAGACGGACCAGAGCGAAA-3′ (forward) and 5′-GGCGGGTCATGGGAATAAC-3′ (reverse). The QuantiTech SYBR Green PCR kit (Qiagen) was used to quantify mRNA levels. Data are normalized to 18S using the comparative C_t method ($\Delta\Delta C_t$).

RNA interference

Tregs or Tconvs were electroporated with 1–6 μM PHLPP1 siRNA (cat. no. M-058853-01 and L-019103-00; Dharmacon), PHLPP2 (cat. no. M-022586-01; Dharmacon), or scrambled control (cat. no. D-001810-01; Dharmacon) using the Nucleofector program X-001.

PHLPP1 overexpression

Tconvs were transfected with pcDNA4/TO-HA or pcDNA4/TO-HA-PHLPP1; transfection efficiency was 37.5 ± 5% after 4 h. Cells were stimulated with plate-bound anti-CD3 (10 μg/ml; 2C11) and soluble CD28 (1 μg/ml; 37.51) in the presence of IL-2 (100 U/ml).

Chromatin immunoprecipitation analysis

Chromatin immunoprecipitation (ChIP) was performed by using Magna ChIP A (Millipore). Tconvs were stimulated with anti-CD3 (10 μg/ml) and anti-CD28 (2 μg/ml) and TGF-β1 (10 ng/ml) for 8 h then fixed with 1% paraformaldehyde. Chromatin was sonicated then incubated with Dynabeads Protein A, saturated with anti–phospho-Smad-3 (Cell Signaling), or rabbit IgG. Immune complexes were washed, and DNA was eluted and analyzed by quantitative PCR using primers from the *PHLPP1* promoter: forward 5′-AGACGGGGCCAGCGATCCTGTGAA-3′ and reverse 5′-GTCGAGGATACCCAGAAGA-3′.

Colitis T cell transfer, histology, and scoring

Colitis was induced in 6- to 10-wk-old male C57Bl6 *TCRβ*$^{-/-}$ mice (The Jackson Laboratory) by i.p. injection of $CD4^+CD25^-CD45RB^{low}$ Tconvs (4×10^5/mouse) alone or together with $CD4^+CD25^{hi}$ Tregs (2×10^5/mouse) from wild-type (WT) or *PHLPP1*$^{-/-}$ mice. Mice were euthanized when weight loss was 10% of initial body weight. Histology and colitis scoring were done as described (15).

Statistical analyses

Paired *t* tests or ANOVA were used for analysis of significance. The *p* values < 0.05 were considered significant and are indicated on the figures accompanying this article as follows unless otherwise indicated: $^*p < 0.05$, $^{**}p < 0.01$. Unless otherwise indicated, error bars represent SD.

Results and Discussion

Tregs express high levels of PHLPP1 and PHLPP2

To investigate the possibility that Tregs express high levels of PHLPP, we sorted $CD4^+Foxp3^-$ and $CD4^+Foxp3^+$ T cells and isolated mRNA. Tregs expressed significantly higher levels of both PHLPP1 (6.9 ± 0.6-fold, $p = 0.0101$) and PHLPP2 (2.3 ± 0.2-fold, $p = 0.0202$) mRNA than those of $CD4^+Foxp3^-$ T cells (Fig. 1*A*). To confirm this finding in humans, $CD4^+CD25^{hi}$ Tregs were sorted from PBMCs and were also found to express high levels of PHLPP1 and PHLPP2 mRNA (6.6 ± 2.3-fold, $p = 0.039$; and 2.2 ± 0.6-fold, $p = 0.0165$, respectively) (Supplemental Fig. 1*A*). Higher expression of PHLPP in human $CD4^+CD25^{hi}$ Tregs was not due to contaminating activated Tconvs, as PHLPP1 mRNA rapidly decreased upon TCR-mediated activation (data not shown). Western blot analysis confirmed increased PHLPP1 protein expression in human Tregs compared with that in Tconvs (Supplemental Fig. 1*B*).

Knockdown of PHLPP restores the activation of Akt in Tregs

To determine if high expression of the PHLPP isozymes directly contributes to the diminished activity of Akt in Tregs, expression of PHLPP1 was reduced using RNA interference. $CD4^+$ T cells were electroporated with PHLPP1 small interfering RNA (siRNA), and expression of PHLPP1 mRNA

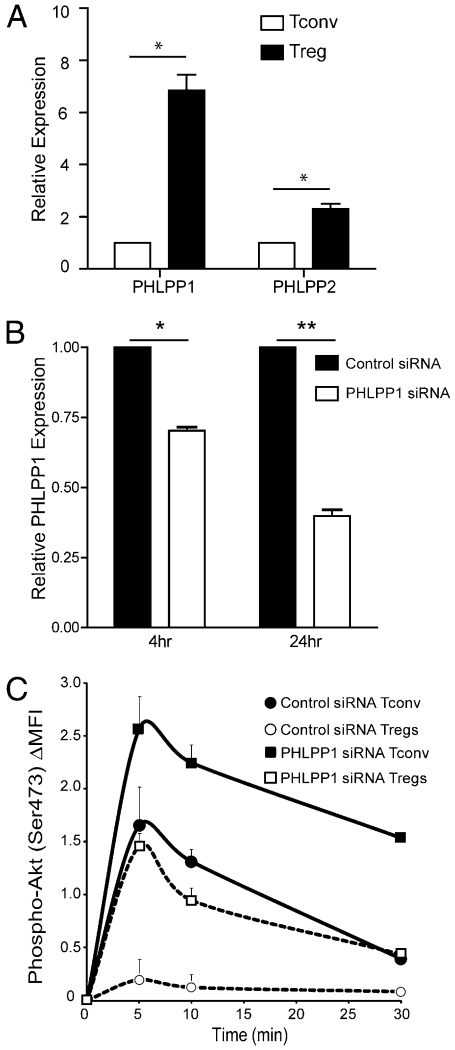

FIGURE 1. High expression of PHLPP in Tregs suppresses the activity of Akt. *A*, PHLPP1 and PHLPP2 mRNA in $Foxp3^-$ Tconvs and $Foxp3^+$ Tregs were quantified by RT-PCR. *B*, $CD4^+$ T cells were transfected with control or PHLPP1 siRNA. The percentage knockdown was 30 ± 4% at 4 h and 60 ± 7% at 24 h. *C*, Twenty-four hours posttransfection, cells were stimulated via the TCR and stained for phospho-Akt in $Foxp3^+$ or $Foxp3^-$ cells. Knockdown of PHLPP1 mRNA resulted in a 7.1-fold increase in Akt activity in Tregs ($p = 0.009$) and a 1.6-fold increase in Tconvs ($p = 0.004$) at 5 min. Data are the average ± SD of three independent experiments. $^*p < 0.05$, $^{**}p < 0.01$.

was reduced by ~55% (Fig. 1*B*). Control or PHLPP1 siRNA-treated T cells were stimulated via the TCR, and Akt activation was determined, gating on Foxp3$^+$ or Foxp3$^-$ cells (15). After TCR stimulation, Akt phosphorylation in CD4$^+$Foxp3$^+$ cells with diminished PHLPP1 expression was restored to levels equivalent to those in control CD4$^+$Foxp3$^-$ T cells (Fig. 1*C*). Notably, diminished expression of PHLPP1 also caused a significant increase in phosphorylation of Akt in CD4$^+$Foxp3$^-$ T cells, indicating this phosphatase has a previously unrecognized role in signal transduction in Tconvs.

We performed similar experiments in human CD4$^+$ T cells. After electroporation with PHLPP1 siRNA and TCR-mediated stimulation, however, there was no detectable change in the phosphorylation of Akt Ser473 (Supplemental Fig. 2*A*). In contrast, when human CD4$^+$ T cells were electroporated with siRNA for both PHLPP1 and PHLPP2 (Supplemental Fig. 2*B*), TCR-mediated activation of Akt in Tregs was completely restored (Supplemental Fig. 2*C*). The differential requirement for PHLPP2 expression in human versus mouse Tregs could be due to a greater capacity of PHLPP2 to compensate for the loss of PHLPP1 in humans. Indeed, in human cells, knockdown of PHLPP1 resulted in greater expression of PHLPP2, and vice versa (Supplemental Fig. 2*D*).

PHLPP1-deficient Tregs remain anergic but have reduced suppressive capacity

We have shown that the hyperactivation of the PI3K pathway reverses the suppressive capacity of Tregs (3) and investigated whether PHLPP1 has a functional role in Tregs by analyzing *PHLPP1*-deficient mice (13). Because T cells had not been previously characterized in *PHLPP1*$^{-/-}$ mice, we first established that peripheral and thymic CD4$^+$Foxp3$^+$ Tregs developed normally at the expected ratios (Supplemental Fig. 3).

Consistent with the siRNA knockdown experiments, in the absence of PHLPP1, TCR-mediated activation of Akt in Tregs was equal to that in Tconvs (Fig. 2*A*). *PHLPP1*$^{-/-}$ Tregs also had restored phosphorylation of a downstream target of Akt, p70-S6K (Fig. 2*B*), indicating that Akt kinase activity is also heightened. PHLPP1 negatively regulates the activity of the MAPK pathway in neurons (16), but the expected diminished activation of ERK in Tregs (17) was not restored in the absence of PHLPP1 (Fig. 2*C*). These data suggest the ability of PHLPP1 to regulate different signaling pathways is cell-type and/or stimulus dependent. We also determined whether the absence of PHLPP1 altered the proliferative and/or suppressive capacity of Tregs. PHLPP1-deficient Tconvs proliferated to the same extent as WT Tconvs (Fig. 2*D*). In the absence

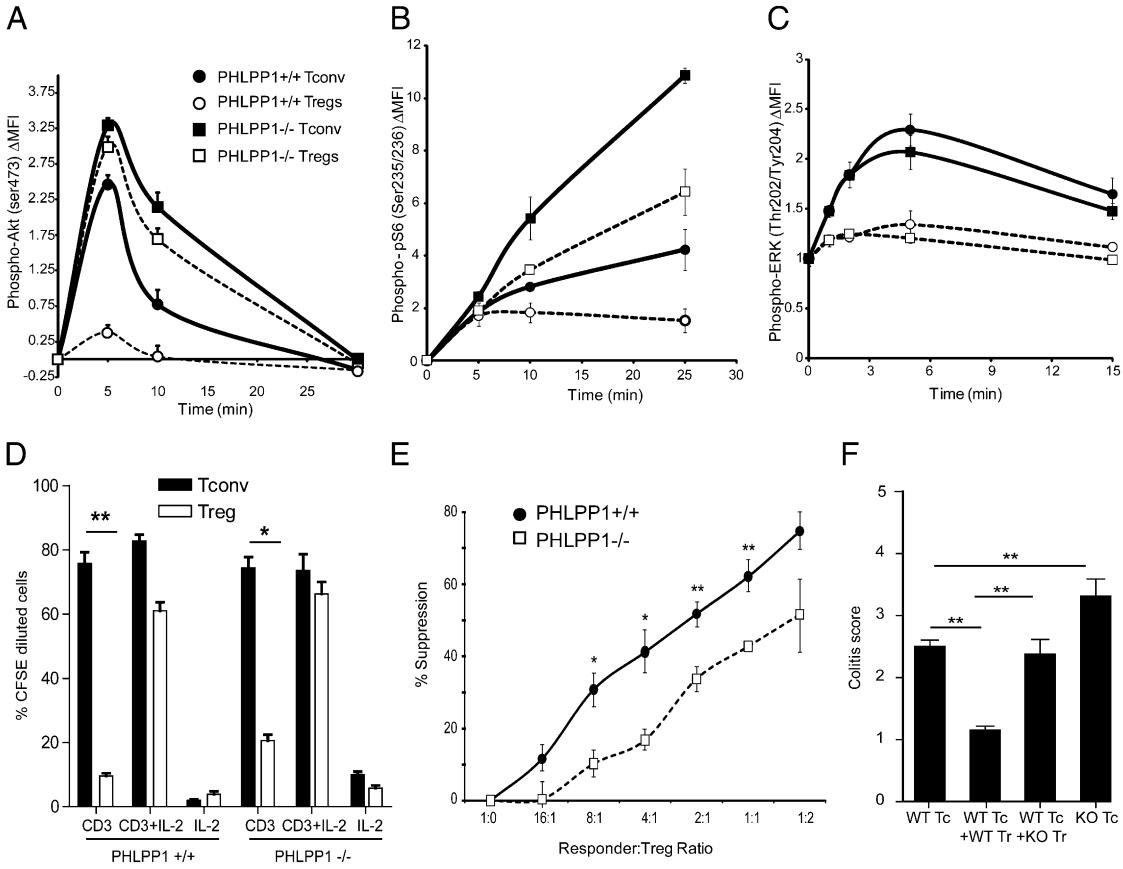

FIGURE 2. *PHLPP1*$^{-/-}$ Tregs have a reduced suppressive capacity. *A–C*, CD4$^+$ T cells from *PHLPP1*$^{-/-}$ and WT mice were stimulated via the TCR, and phosphorylation of Akt, p70 S6 kinase, and ERK was determined in Foxp3$^+$ and Foxp3$^-$ cells. Foxp3$^+$ cells from *PHLPP1*$^{-/-}$ mice had a 7.9-fold increase in Akt activity ($p = 0.002$) compared with a 1.3-fold increase in Foxp3$^-$ Tconvs ($p = 0.014$). *D*, CFSE-labeled *PHLPP1*$^{-/-}$ or WT Tregs and Tconvs were stimulated with anti-CD3 mAbs in the absence or presence of IL-2. The percentage of cells that diluted CFSE is depicted. *E*, CFSE-labeled WT CD4$^+$ T cells were stimulated with anti-CD3 mAbs and APCs with increasing numbers of Tregs from WT or *PHLPP1*$^{-/-}$ mice. Data depict averages ± SD from three independent experiments. *F*, TCRβ$^{-/-}$ mice were injected with Tconv (Tc) alone or coinjected with Tregs (Tr) from WT or *PHLPP1*$^{-/-}$ (KO) mice. Mice were sacrificed when the groups without Tregs lost 10% of body weight. Proximal and distal colon sections were scored blindly by two investigators, and results are the mean colitis score from five mice per group. $^*p < 0.05$, $^{**}p < 0.001$.

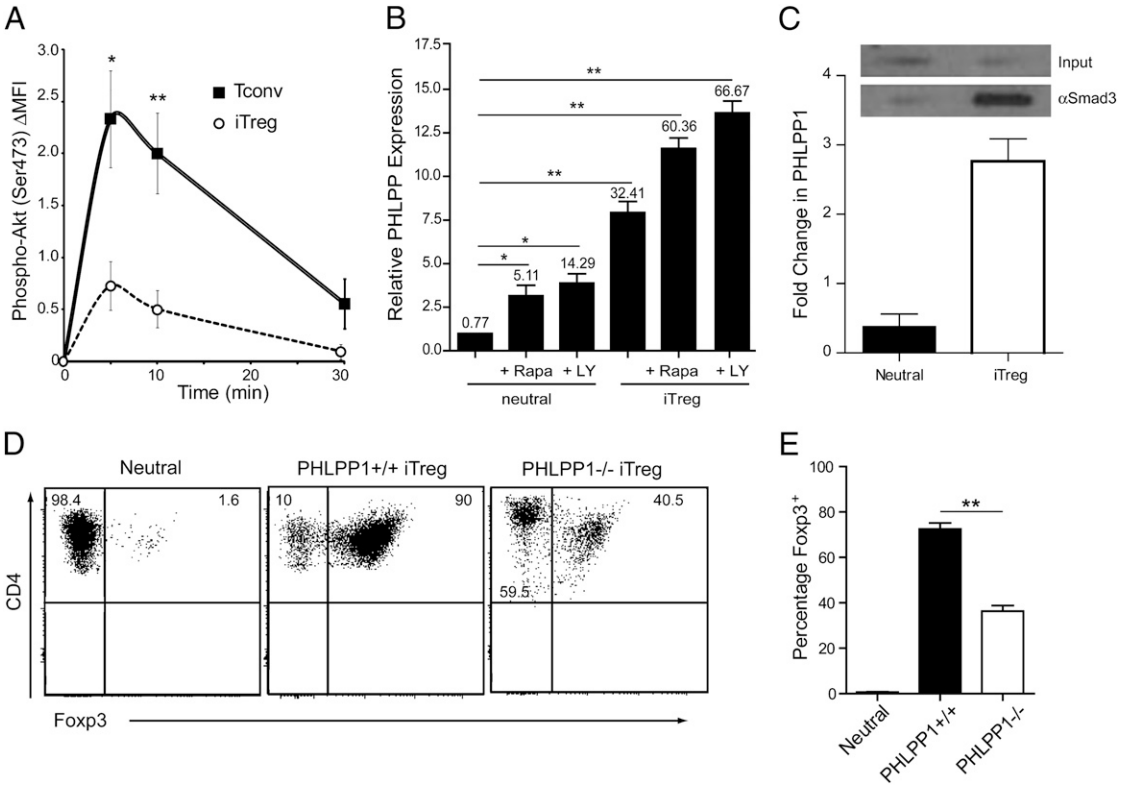

FIGURE 3. Expression of PHLPP1 is required for the development of iTregs. Tconvs were stimulated the absence (Tconv) or presence (iTreg) of TGF-β. *A*, After 4 d, cells were restimulated via the TCR, and levels of phospho-Akt were determined. *B*, Tconvs were stimulated as above without or with LY294002 or rapamycin. After 4 d, the proportion of Foxp3$^+$ cells and PHLPP1 mRNA expression was determined. Numbers above bars indicate the percentage of Foxp3$^+$ cells ($n = 5$, mean ± SEM). *C*, Tconvs were stimulated as above for 8 h. Chromatin was isolated and subjected to ChIP analysis with anti–phospho-Smad-3 Abs. Bar graph indicates data normalized against input DNA. *D* and *E*, Tconvs from *PHLPP1*$^{-/-}$ or *WT* mice were stimulated as above, and after 4 d the proportion of Foxp3-expressing cells was determined. *A*, *B*, *C*, and *E* depict averaged data from 4, 5, 2, and 5 independent experiments, respectively, and *D* depicts a representative experiment. $^*p < 0.05$, $^{**}p < 0.001$.

of IL-2, Tregs from both genotypes failed to proliferate, indicating that PHLPP1 is not required for Treg anergy (3). In contrast to PTEN-deficient Tregs (18), PHLPP1-deficient Tregs did not proliferate in response to IL-2 alone, indicating a distinct role for these two phosphatases in regulation of cytokine-stimulated growth.

We next purified Tregs from *PHLPP1*$^{-/-}$ and WT littermates and compared their capacity to suppress WT CD4$^+$ T cells. PHLPP1-deficient Tregs were significantly less suppressive than the WT controls (Fig. 2*E*, Supplemental Fig. 4*A*). This was also true in vivo, as PHLPP1-deficient Tregs were unable to protect from colitis induced by naive Tconvs (Fig. 2*F*). Mice receiving *PHLPP1*$^{-/-}$ Tconvs had enhanced colitis compared to mice receiving WT Tconvs (Fig. 2*F*, Supplemental Fig. 4*B*). Moreover, *PHLPP1*$^{-/-}$ Tconvs were less susceptible to suppression by WT Tregs in vitro (Supplemental Fig. 4*C*), supporting the notion that increased activity of Akt in Tconvs makes them less susceptible to suppression by Tregs (5, 19).

PHLPP1 expression is required for the development of induced Foxp3$^+$ Tregs

The PI3K/Akt pathway also regulates the conversion of Tconvs into induced regulatory T cells (iTregs) (5). To investigate whether iTregs have a defect in activation of Akt, Tconvs were stimulated with TGF-β and IL-2 to induce Foxp3 expression (15, 20), then stimulated via the TCR. iTregs displayed a significant defect in activation of Akt (Fig. 3*A*). Moreover, on average, iTregs induced by TGF-β expressed 8-fold more PHLPP1 mRNA than Tconvs (Fig. 3*B*). In addition to TGF-β, blockade of the PI3K pathway with pharmacological inhibitors can also induce Foxp3 and iTregs (4). Similar to

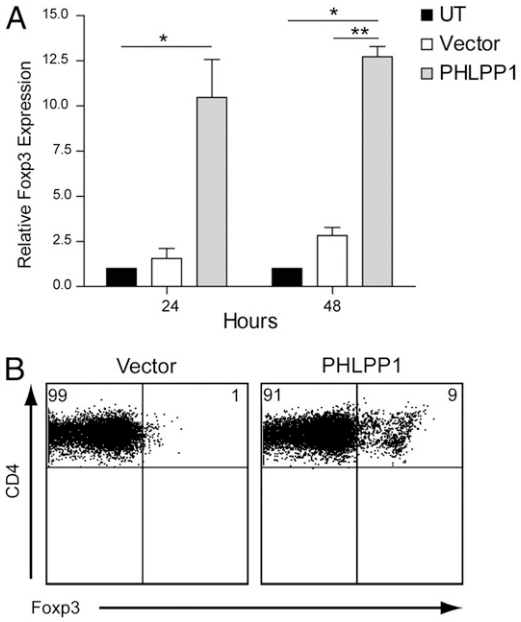

FIGURE 4. Overexpression of PHLPP1 induces Foxp3 expression. CD4$^+$ Tconvs were transfected with a control or PHLPP1-expressing vector. *A*, After 24- or 48-h expression of Foxp3, mRNA was determined. *B*, After 48-h expression of Foxp3, protein was determined. *A* depicts averaged data from two independent experiments, and *B* depicts one representative experiment of three. $^*p < 0.05$, $^{**}p < 0.001$.

treatment with TGF-β, stimulation in the presence of either LY294002 (which inhibits PI3K) or rapamycin (an inhibitor of the mTOR kinase when part of the mTORC1 complex) induced expression of PHLPP1 in parallel to Foxp3 (Fig. 3*B*). Indeed, there was a strong correlation between expression of PHLPP and Foxp3, suggesting that upregulation of PHLPP1 may be part of the Treg developmental program.

TGF-β–induced Foxp3 expression requires activation of Smad-3 (21), and analysis of the PHLPP1 promoter revealed nine putative Smad-3 binding sites. To test if Smad-3 binds to the PHLPP promoter, we performed ChIP analysis with Tconvs stimulated under neutral or iTreg differentiation conditions. We detected a significant increase in binding of phospho-Smad-3 to the PHLPP1 promoter in the presence of TGF-β (Fig. 3*C*) suggesting that PHLPP expression is regulated by this immunosuppressive cytokine.

To test directly if expression of PHLPP was required for the development of iTregs, Tconvs from WT and *PHLPP1*$^{-/-}$ mice were stimulated with TGF-β and IL-2, and after 4 d the proportion of Foxp3^{+} cells was analyzed. On average, PHLPP1-deficient T cells had a 49.6 ± 3.1% (p = 0.002) reduction in the intracellular expression of Foxp3 (Fig. 3*D*, 3*E*, Supplemental Fig. 5*A*). We also knocked down the expression of PHLPP2 in *PHLPP1*$^{-/-}$ Tconvs and found there was an almost complete block in iTreg development (Supplemental Fig. 5*B*). Thus, as in humans, PHLPP2 may compensate for the absence of PHLPP1 in mice.

Conversely, transient overexpression of PHLPP1 induced de novo expression of Foxp3 mRNA and protein (Fig. 4), indicating that this expression of this phosphatase is sufficient for the expression of Foxp3, at least in a subset of cells. The finding that overexpression of PHLPP1 promotes expression of Foxp3 is consistent with the notion that blockade of the PI3K pathway is essential for iTreg development (4, 5, 22) and provides molecular evidence for how TGF-β may feed into this pathway by upregulating PHLPP1 expression.

Despite the defect in development of iTregs, *PHLPP1*$^{-/-}$ mice appear to have normal development of natural Tregs, as we detected neither a decrease in the number of thymic or circulating Foxp3^{+} cells nor a decrease in the intensity of Foxp3 expression. It is possible that PHLPP2 may compensate for PHLPP1 in these mice, or that the development of natural Tregs is less reliant on a finely-tuned PI3K pathway compared to iTregs. In support of the latter possibility, mice lacking mTOR have a similar phenotype: development of natural Tregs was unaffected despite differences in the development of iTregs (22).

In conclusion, high expression of PHLPP restrains Akt activity in Tregs, ultimately allowing their normal development and function. This study provides insight into the molecular regulation of Tregs and establishes a new paradigm for how the PI3K pathway is negatively regulated in T cells. Our data are consistent with the notion that reduced Akt activity is a functional requirement for Tregs and with the hypothesis that high expression of PHLPP is a key molecular regulator of this pathway in Tregs. Further research will be required to define how the expression and activity of PHLPP is regulated in Tregs, whether PHLPP may control the activity of other signaling molecules, such as protein kinase C family members, in addition to PI3K in Tregs, and whether there may be environmental contexts in which Tregs are more or less reliant on this molecule to exert their effects.

Disclosures

The authors have no financial conflicts of interest.

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