Abstract

Despite the strides that immunotherapy has made in mediating tumor regression, the clinical effects are often transient, and therefore more durable responses are still needed. The temporary nature of the therapy-induced immune response can be attributed to tumor immune evasion mechanisms, mainly the effect of suppressive immune cells and, in particular, regulatory T cells (Treg). Although the depletion of Tregs has been shown to be effective in enhancing immune responses, selective depletion of these suppressive cells without affecting other immune cells has not been very successful, and new agents are sought. We found that PI3K–Akt pathway inhibitors selectively inhibit Tregs with minimal effect on conventional T cells (Tconv). Our results clearly show selective in vitro inhibition of activation (as represented by a decrease in downstream signaling) and proliferation of Tregs in comparison with Tconvs when treated with different Akt and PI3K inhibitors. This effect has been observed in both human and murine CD4 T cells. In vivo treatment with these inhibitors resulted in a significant and selective reduction in Tregs in both naïve and tumor-bearing mice. Furthermore, these PI3K–Akt inhibitors led to a significant therapeutic antitumor effect, which was shown to be Treg dependent. Here, we report the use of PI3K–Akt pathway inhibitors as potent agents for the selective depletion of suppressive Tregs. We show that these inhibitors are able to enhance the antitumor immune response and are therefore promising clinical reagents for Treg depletion. Cancer Immunol Res; 2(11); 1080–9. ©2014 AACR.

Introduction

Cancer immunotherapy has proved successful in mediating disease regression in patients with cancer. Several strategies have been used effectively to generate therapeutic tumor antigen-reactive T-cell responses. These include active immunization (1–5), adoptive cell transfer (ACT) of lymphocytes genetically engineered for antitumor function (6–9), and ACT of tumor-infiltrating lymphocytes (TIL; refs. 10–16). Despite these successes, many responses are transient, and improvements are needed to increase durable responses in patients.

A suppressive tumor immune environment is thought to limit the efficacy of ACT and active immunization approaches. Chief among the suppressive cells are CD4 regulatory T cells (Treg) that can be distinguished by the expression of FoxP3 and CD25HI molecules (17). An opposing action of Tregs and cytotoxic CD8 T cells in tumor regression has been identified in mouse models (18). A correlation of tumor-infiltrating Tregs with poor clinical prognosis has been demonstrated in humans (19–22). In fact, the depletion of Tregs was found to enhance antitumor immunity and promote tumor regression in mouse models (23–26).

Despite the evidence of the suppressive role of Tregs in the tumor microenvironment (TME), there is still a paucity of Treg-depleting clinical reagents. Concerns over efficacy and specificity have burdened currently available clinical reagents such as low-dose cyclophosphamide and the anti-CD25 monoclonal antibody (mAb) daclizumab (27). Novel clinical reagents that specifically target Treg persistence and survival are candidates for tumor immunomodulation.

Several reports have shown that Tregs and conventional T cells (Tconv) display unique signaling signatures downstream of the T-cell receptor (TCR; refs. 28–31). The PI3K–Akt pathway plays a critical role in the cellular response to TCR engagement and costimulation (32, 33). Active PI3K–Akt signaling results in increased cytokine gene expression, a characteristic of productive T-cell activation (34). In addition to transmitting signals critical for T-cell activation, many T-cell functions are governed by PI3K–Akt signaling, which includes proliferation, survival, migration, and metabolism (35, 36).

Because of the important role of the PI3K–Akt pathway in T-cell function (35, 36) and the reported differences between Tregs and Tconvs downstream of the TCR (28–31), we evaluated the effect of PI3K–Akt inhibition on Tregs and Tconvs. The impact of Akt and PI3K inhibitors on Treg and Tconv...
activation and proliferation is assessed in vitro. We have also evaluated the effect and therapeutic efficacy of in vivo treatment with these inhibitors on the antitumor immune response.

Materials and Methods

**Mice and cell lines**

Female C57BL/6J-H-2b and BALB/c mice (6–10-week-old; NCI, Frederick, MD) were housed under pathogen-free conditions. All procedures were carried out with approved institutional animal protocols. B16, CT26, and EL4 cell lines were obtained from the ATCC, which routinely authenticate and test these cell lines (for *Mycoplasma*, by the Hoechst stain, PCR, and the standard culture test). These cells were used within 6 months of purchase. TC-1 (established by immortalization with the HPV16 E6 and E7 genes and its growth enhanced by Tregs; refs. 37, 38) was a gift from Prof. T.C. Wu (Johns Hopkins University, Department of Pathology, Baltimore, MD). These cells, along with B16, were authenticated and tested for mouse parvovirus (MPV) and mouse hepatitis virus (MHV) using PCR at Georgia Regents University (Augusta, GA). All tests were negative.

**Reagents**

The PI3K inhibitor wortmannin and the Akt inhibitor triciribine were obtained from Calbiochem. IC87114, a PI3K inhibitor, and MK-2206, an Akt inhibitor, were purchased from SelleckChem. The 9-mer synthetic peptide from HPV16 E7 (57, RAHYNIVTF, was obtained from Celske Bioscience. E7 (100 μg/mouse; Peprotech), anti-CD40 (20 μg/mouse; BioLegend), and Incomplete Freund Adjuvant (IFA; 50 μL/mouse; Sigma). This was reported as the most effective therapeutic combination for this vaccine (39).

**Human T-cell cultures**

Leukapheresis products were obtained from healthy human donors (Department of Transfusion Medicine, NIH, Bethesda, MD). Peripheral blood mononuclear cells (PBMC) were prepared over ficoll-paque plus gradient centrifugation (GE Healthcare), and CD4+CD25+ and CD4+CD25- cells were sorted using the FACSAria II flow cytometer. The cells were then labeled with CFSE (Life Technologies) according to the manufacturer’s instructions. Fifty thousand cells were cultured with anti-CD3/CD28–conjugated Dynabeads (Life Technologies) at a 1:1 cell-to-bead ratio in RPMI-1640 supplemented with 5% autologous serum and 100 U/mL IL2 (PeproTech) for 3 days, in the presence or absence of escalating concentrations of inhibitors. CFSE dilution was then assessed by flow cytometry.

**Murine CD4 T-cell cultures**

Magnetic bead purification kits (Miltenyi Biotec) were used to enrich CD4+CD25- and CD4+CD25+ T cells from murine splenocytes following the manufacturer’s instructions. Cells were labeled with CFSE (Life Technologies) and cultured in 24-well plates at a density of 5 × 10^5 cells per well in RPMI-1640 (Life Technologies) with 10% FCS in the presence of 10 μg/mL plate-bound anti-CD3 (BD Biosciences), 1 μg/mL soluble anti-CD28 (BD Biosciences), and 100 IU/mL IL2 (R&D Systems). Plates were centrifuged and then incubated at 37°C, 5% CO2, for 72 hours. Wortmannin (200 nmol/L), MK-2206 (2 μmol/L), IC87114 (10 μmol/L), or DMSO (carrier) were added to the culture media from the beginning. CFSE dilution was measured by flow cytometry.

The phosphorylation level of S6 was assessed. Murine cells were prepared as described above and stimulated for 15 minutes. Thirty micrograms of cell lysates in RIPA buffer was then run on SDS-PAGE gels, transferred to polyvinylidene fluoride (PVDF) membranes probed with primary antibodies (1:1,000 anti-pS6 and anti-S6; Cell Signaling Technology) overnight at 4°C, and incubated with secondary antibodies (1:2,000) for 1 hour at room temperature. Chemiluminescence was performed with Pierce reagents, and densitometric analysis was performed using ImageJ (NIH).

**In vivo experiments to assess splenocyte composition**

Tumor-free naïve mice were injected intraperitoneally (i.p.) with 40 μg of wortmannin, 50 μg of triciribine, or 10 μg of MK-2206 dissolved in 35% DMSO in PBS (100-μL volume). Mice were injected every other day for a week. Splenocytes were then harvested, and CD4+ and Foxp3+ composition was assessed by flow cytometry.

**IFNγ ELISPOT**

Tumor-free naïve mice were injected i.p. on alternate days with 40 μg of wortmannin, 50 μg of triciribine, or DMSO vehicle for a week before vaccination with E7 vaccine (E7 39-47, GM-CSF, anti-CD40, and IFA), which was given subcutaneously (s.c.) on days 7 and 14. One week after the second E7 vaccination, splenocytes were harvested, and the anti-E7 immune response was assayed by peptide restimulation and IFNγ ELISPOT (BD Biosciences), according to the manufacturer’s instructions.

**Tumor treatment**

C57BL/6 female mice were implanted with 50,000 TC-1 cells per mouse s.c. in the right flank on day 0. The mice were then treated with 40 μg of wortmannin or DMSO for 1 week after palpable tumors were detected. Mice were then vaccinated with E7 peptide vaccine (E7 49-57, GM-CSF, anti-CD40, and IFA), as described above, and tumor growth was monitored. The same model was used with MK-2206, in which mice were challenged with TC-1 cells on day 0, and on days 7 and 14, mice in the appropriate groups were injected with the E7 vaccine and/or MK-2206 (30 μg). The tumors were measured and the mice were euthanized on day 21. The tumor immune infiltrate was then assessed by flow cytometry. A prophylactic therapeutic model was also used without vaccines. Three tumor models were used: B16 (200,000 cells/mouse, s.c.) and EL4 (100,000 cells/mouse s.c.), both in C57BL/6 mice, and CT26 (500,000 cells/mouse, s.c.) in BALB/c mice. Mice were treated with 40 μg of wortmannin, 50 μg of triciribine, or DMSO (i.p.) on days −7, −4, and −2 before s.c. tumor inoculation on day 0, and tumor growth was monitored thereafter. Ex vivo–activated Tregs (cells were stimulated as described above for 72 hours) were infused i.v. (10,000 cells/mouse) into wortmannin-treated EL4 and CT26 tumor-bearing animals on day 4 after
tumor inoculation, and tumor growth was also monitored. Foxp3+ T-cell infiltration into CT26 tumor was assessed by flow cytometry on days 20 and 24 following tumor inoculation.

Flow cytometry analysis
Staining for surface markers (CD4 and CD8) was performed using mAbs (BD Biosciences). Cells were incubated for 20 minutes on ice in PBS, 2% BSA, and 0.1% sodium azide. Intracellular staining kits were used to stain for Foxp3 using anti-Foxp3-PE mAb (eBioscience). Data acquisition was performed on FACSCalibur or FACSscan cytometers (BD Biosciences). Results were analyzed with CellQuest (BD Biosciences), WinMDI (Purdue University, West Lafayette, IN), or FlowJo (TreeStar).

Statistical analysis
All statistical parameters (average values, SD, significant differences between groups) were calculated using GraphPad Prism software. Statistical significance between groups was determined by a paired t test or ANOVA with a post hoc Tukey multiple comparison test (P < 0.05 was considered statistically significant).

Results

Human CD4 Tregs are more dependent on PI3K–Akt signaling for TCR-induced proliferation than conventional CD4 T cells
Because of the vital role that the PI3K–Akt pathway plays in many T-cell functions, including proliferation and survival, we evaluated the effect of inhibiting this pathway on human CD4+ T-cell proliferation. CD4+ Tregs were identified as CD4+ CD25+ Foxp3+, whereas conventional CD4+ Tconvs were identified as CD4+ CD25-. Tregs and Tconvs were fractionated from human PBMCs by fluorescence-activated cell sorting. Proliferation was examined by dilution of CFSE after stimulation with anti-CD3/anti-CD28 in a 3-day culture in media containing 100 IU/mL IL2 with wortmannin, MK-2206, or IC87114 (triciribine was found to be toxic to murine T cells in these prolonged in vitro culture conditions). Similar to results obtained with human CD4 T-cell subsets, data from at least two independent experiments demonstrated that mouse Tregs were significantly more sensitive to proliferative inhibition by wortmannin (200 nmol/L), IC87114 (10 µmol/L), and MK-2206 (2 µmol/L) than Tconvs (Fig. 1A).

Inhibition of PI3K–Akt signaling by inhibitors in Tregs was also confirmed by Western blot analysis, in which phosphorylation of S6 was used to identify active signaling through the PI3K–Akt pathway. Conventional CD4 T-cell subsets using magnetic bead enrichment kits. After enrichment, Tregs and Tconvs were assayed for viability by 7-AAD incorporation. Culture viability was inversely related to the amount of inhibitor present across the dose range used; however, the viability was reduced equally in Tregs and Tconvs. This was observed with all inhibitors (wortmannin, IC87114, triciribine, and MK-2206; data not shown).

Taken together, our data demonstrate that human Tregs are more dependent on the PI3K–Akt signaling pathway for proliferation in response to anti-CD3/anti-CD28/IL2 stimulation compared with Tconvs.

PI3K–Akt pathway is necessary for CD4 Treg maintenance in mice
The documented dependence of murine T-cell proliferation and other vital functions on the PI3K–Akt pathway, in addition to the necessity of this pathway in human Treg proliferation shown above, prompted a similar evaluation in murine Treg proliferation. Mouse CD4+ splenocytes were fractionated into CD25+ (Treg) and CD25- (Tconv) subsets using magnetic bead enrichment kits. After enrichment, Tregs and Tconvs were >92% and >95% pure, respectively, based on CD4 and Foxp3 expression (data not shown). Proliferation was assayed by dilution of CFSE after stimulation with anti-CD3/anti-CD28 and 3-day culture in media containing 100 IU/mL IL2 with wortmannin, MK-2206, or IC87114 (triciribine was found to be toxic to murine T cells in these prolonged in vitro culture conditions). Similar to results obtained with human CD4 T-cell subsets, data from at least two independent experiments demonstrated that mouse Tregs were significantly more sensitive to proliferative inhibition by wortmannin (200 nmol/L), IC87114 (10 µmol/L), and MK-2206 (2 µmol/L) than Tconvs (Fig. 2A).

Inhibition of PI3K–Akt signaling by inhibitors in Tregs was also confirmed by Western blot analysis, in which phosphorylation of S6 was used to identify active signaling through the PI3K–Akt pathway. Confirming the inhibition of PI3K–Akt signaling, treatment of anti-CD3/anti-CD28/IL2–stimulated Tregs with wortmannin, triciribine, IC87114, or MK-2206 resulted in a marked decrease of phosphorylated S6 in comparison with Tconvs (Fig. 2B and C).

As a result of the significant in vitro difference between the proliferation inhibition of Tregs and Tconvs by PI3K and Akt inhibitors, the role of PI3K–Akt signaling in the in vivo cellular composition of spleens was examined. In these analyses, mice were treated with wortmannin (40 µg), triciribine (50 µg), MK-2206 (10 µg), or DMSO for 1 week on alternate days before flow cytometry analysis of their splenocyte composition. Mice treated with DMSO contained a percentage of CD4 T cells similar to wortmannin-, triciribine-, or MK-2206–treated mice (Fig. 3A). Similarly, spleens of DMSO and inhibitor-treated mice exhibited no differences in the percentages of CD8 T cells (data not shown). Importantly, despite not affecting total CD4 T cells, the percentage of Foxp3+ cells within the CD4+ T-cell population was significantly reduced in mice treated with wortmannin, triciribine, or MK-2206 (Fig. 3B).

The in vitro dependence of Treg proliferation on the PI3K–Akt pathway, coupled with the in vivo reduction in the actual numbers of these suppressive cells in response to PI3K and Akt inhibition, led us to evaluate the effect of these inhibitors on...
Figure 1. The inhibition of PI3K and Akt in human T cells selectively inhibits the proliferation of human Tregs compared with Tconvs in a dose-dependent manner. Tregs (CD4^+CD25^Hi) and Tconvs (CD4^+CD25^-/Lo) were fractionated from human PBMCs by cell sorting. Proliferation was examined by dilution of CFSE after stimulation with anti-CD3/anti-CD28 for 3 days with titrated amounts of triciribine (TCN), MK-2206, wortmannin (WM), and IC87114. Dead cells were excluded by 7-AAD incorporation. Left, average of three experiments, normalized to untreated controls; right, representative examples. A, a significant reduction in Treg proliferation was observed in response to Akt inhibition by triciribine compared with that of Tconvs at all doses tested: 5 \( \mu \text{mol/L} \) (\( P = 0.04 \)), 10 \( \mu \text{mol/L} \) (\( P = 0.03 \)), and 50 \( \mu \text{mol/L} \) (\( P = 0.01 \)). B, a significant reduction of Treg proliferation in response to MK-2206 treatment was observed compared with that of Tconvs at the 1 \( \mu \text{mol/L} \) (\( P = 0.05 \)) and 5 \( \mu \text{mol/L} \) (\( P = 0.005 \)) doses. C, proliferation of Tregs was significantly reduced by wortmannin compared with that of Tconvs at all doses tested: 300 nmol/L (\( P = 0.03 \)), 800 nmol/L (\( P = 0.01 \)), and 1,000 nmol/L (\( P = 0.002 \)). D, Treg proliferation was significantly reduced by IC87114 compared with that of Tconvs at the 10 \( \mu \text{mol/L} \) (\( P = 0.04 \)) and 20 \( \mu \text{mol/L} \) (\( P < 0.005 \)) doses. * \( P < 0.05 \).
Treg immunosuppressive ability in vivo. Mice were conditioned on alternate days with wortmannin, triciribine, or DMSO for a week before vaccination with HPV16 E7 peptide-based vaccine that was given s.c. on days 7 and 14. One week after the second vaccination, splenocytes were harvested, and the E7-specific immune response was assayed by IFNγ ELISPOT after restimulation with E7 peptides. As expected, vaccination-induced E7-reactive T cells, and importantly addition of wortmannin and triciribine to vaccine treatment, significantly increased the number of E7-reactive T cells compared with those of the controls (Fig. 3C).

Taken together, these data demonstrate that the PI3K–Akt pathway is necessary for Treg proliferation, FoxP3+ CD4+ Treg maintenance in mice, and inhibition of this pathway resulted in an augmented T-cell response to peptide vaccination otherwise suppressed by the presence of Tregs.

PI3K–Akt inhibition mitigates tumor growth

The depletion of Tregs results in enhanced antitumor immune responses (23). Our data so far show that PI3K and Akt inhibition results in selective in vitro inhibition of Tregs and enhanced immune response to peptide vaccination. We therefore tested the utility of targeting the PI3K–Akt pathway for tumor treatment. Mice implanted s.c. with TC-1 tumor cells were treated with wortmannin or DMSO for 1 week after palpable tumors were detected. Mice were then vaccinated with the E7 vaccine as described above and tumor growth was monitored. Although both E7 vaccination and wortmannin treatment significantly inhibited TC-1 tumor growth (P < 0.05 and P < 0.001, respectively), the greatest impairment was achieved with the combination of wortmannin treatment and E7 vaccination (P < 0.0001; Fig. 4A).

To evaluate the mechanism by which PI3K–Akt inhibition reduced tumor growth and to minimize the direct effect of PI3K–Akt inhibitors on the tumors, a prophylactic tumor model was employed using B16 and EL4 tumor cells. Mice were treated with wortmannin, triciribine, or DMSO on days −7, −4, and −2 before s.c. tumor inoculation on day 0, and tumor growth was monitored thereafter. Wortmannin and triciribine were chosen because of their short half-lives (10 minutes and 6 hours, respectively) to ensure a minimal direct cytotoxic effect of the inhibitors on the tumor cells. Tumor growth was significantly inhibited by wortmannin and triciribine compared with DMSO in the B16 (P < 0.0001; Fig. 4B) and EL4 (data not shown) tumor models.
Foxp3$^+$ Tregs Are Dependent on the PI3K–Akt Pathway

These results demonstrate the effectiveness of PI3K and Akt inhibitors in suppressing tumor growth even without a tumor-specific vaccine and with minimal direct effect on the tumor cells.

**Inhibition of the PI3K–Akt pathway enhances the antitumor effect of tumor-specific vaccines**

The above results clearly show an enhanced antitumor effect of a vaccine when combined with PI3K–Akt pathway inhibitors. To further evaluate the effect of PI3K–Akt pathway inhibition on the TME, we tested the effect of combining the E7 vaccine with MK-2206 on tumor growth and immune-cell infiltration in the TC-1 mouse tumor model. MK-2206 is currently in several clinical trials and has a long half-life (60–90 hours), and was therefore chosen to be used to assess the effect of Akt inhibition on the TME. Following tumor implantation (day 0), appropriate groups of mice were injected with the E7 vaccine and/or MK-2206. Tumors were measured and the mice were euthanized on day 21, and the intratumoral lymphocytic infiltrate was assessed. Both the vaccine and MK-2206 individually significantly reduced tumor sizes in comparison with the nontreated controls ($P < 0.05$; Fig. 5A); however, when combined, they reduced the tumor volume more profoundly compared with that of nontreated controls ($P < 0.01$) and of single vaccine treatment ($P < 0.05$).

The tumor tissues were then processed, and the numbers of CD8$^+$ and Foxp3$^+$ T cells per million tumor cells within the tumors were evaluated. In tumors treated with the combination of MK-2206 with the vaccine, a significantly higher number of CD8$^+$ T cells were detected in comparison with that of the nontreated group ($P < 0.001$; Fig. 5B). The group treated with the combination also showed a significant reduction in Treg (Foxp3$^+$) infiltration in comparison with that of the nontreated group ($P < 0.05$) and the vaccine alone group ($P < 0.01$; Fig. 5C).

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**Figure 3.** The PI3K–Akt pathway is essential for the in vivo maintenance of murine Tregs. Mice were conditioned with wortmannin (WM; 40 μg), triciribine (TCN; 50 μg), MK-2206 (10 μg), or DMSO for 1 week on alternate days before flow cytometric analysis of splenocytes. Alternatively, mice were conditioned on alternate days with either wortmannin or DMSO for a week before vaccination with E7. One week after E7 vaccination, splenocytes were harvested, and the E7 immune response was assayed by ELISPOT. A, mice treated with DMSO vehicle contained similar percentages of CD4$^+$ T cells compared with wortmannin-, triciribine-, or MK-2206–treated animals ($P = 0.1$). B, the number of FoxP3$^+$ cells in CD4$^+$ T cells was significantly reduced in the mice treated with the inhibitors compared with DMSO ($P < 0.05$). C, vaccination resulted in a significant increase in E7-reactive T cells. Wortmannin and triciribine treatment significantly increased the number of E7-reactive cells compared with DMSO-treated controls. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

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**Figure 4.** PI3K–Akt signaling inhibition mitigates tumor growth. A, TC-1 tumor-bearing mice were treated with wortmannin (WM) or DMSO for 1 week after palpable tumors were detected. Mice were then vaccinated with E7 peptide and tumor growth was monitored. Both E7 vaccination ($P < 0.05$) and wortmannin treatment ($P < 0.001$) significantly inhibited TC-1 tumor growth, yet the greatest impairment was achieved with the combination of wortmannin treatment and E7 vaccination ($P < 0.0001$). B, mice were prophylactically treated with wortmannin, triciribine (TCN), or DMSO before s.c. B16 tumor inoculation and tumor growth was monitored. Tumor growth was significantly inhibited by wortmannin and triciribine compared with DMSO-treated animals ($P = 0.0001$).

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**Table 1.** Summary of effects of PI3K and Akt pathway inhibitors on tumor growth and immune-cell infiltration. A, 60 days after tumor inoculation. B, 90 days after tumor inoculation.

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These data further emphasize the enhancement of the therapeutic efficacy of tumor vaccines by the use of an Akt inhibitor and demonstrate an augmentation of the antitumor immune response when the vaccine was combined with the inhibitor.

**PI3K–Akt inhibition reduces tumor growth in a Treg-dependent manner**

So far, we have shown that inhibiting the PI3K–Akt pathway results in the suppression of tumor growth, enhancement of the therapeutic efficacy of tumor-specific vaccine, and enhancement of the antitumor immune response (increase in intratumoral CD8\(^+\) cells and decrease in Tregs). To confirm the role of Treg depletion by PI3K–Akt inhibitors in their antitumor therapeutic effect, the CT26 tumor model was used. Similar to what was observed with the B16 and EL4 tumor models, prophylactic treatment of mice with wortmannin or triciribine before s.c. tumor inoculation resulted in a significant tumor growth inhibition by triciribine \((P < 0.0001; \text{Fig. 6A})\) and wortmannin \((P < 0.0001; \text{Fig. 6B})\) compared with that in DMSO-treated animals. However, this effect was reversed when the wortmannin-treated mice were reconstituted with Tregs. \textit{Ex vivo}–cultured Treg infusion on day 4 resulted in the restoration of tumor growth, comparable with that in DMSO-treated mice (Fig. 6B). The same effect was observed with the EL4 tumor model (data not shown).

An analysis of FoxP3\(^{+}\) T-cell infiltration into CT26 tumors elucidated a significant decrease in Treg numbers after wortmannin treatment that was restored after infusion of \textit{ex vivo}–grown Tregs 4 days after tumor inoculation (Fig. 6C).

Taken together, these data demonstrate that the Treg dependence on PI3K–Akt signaling can be exploited to selectively deplete suppressive CD4 Tregs, resulting in an enhanced capacity to elicit an antitumor immune response when combined with the vaccine.

**Discussion**

CD4 T cells differentiate into a panoply of effector cells with an array of diverse functions in the immune response. Preclinical mouse models have identified inflammatory CD4 T cells that mediate tumor regression and CD4 Tregs that support tumor growth (18).

A correlation of tumor-infiltrating Tregs with poor clinical prognosis has been demonstrated (19–22), and the depletion of Tregs was found to enhance antitumor immunity and promote tumor regression (23–26). However, there is still a scarcity of efficient and highly selective Treg-depleting clinical reagents for use in tumor immunomodulation.

Several reports have shown that Tregs and Tconvs display unique signaling signatures downstream of TCR (28–31). The PI3K–Akt signaling pathway is important in the cellular response to TCR stimulation and costimulation (32, 33). This pathway plays a critical role in T-cell functions, including proliferation, survival, migration, and metabolism (35, 36).

Because of the important role of the PI3K–Akt pathway in T-cell function (35, 36) and the reported differences between Tregs and Tconvs downstream of TCR (28–31), we investigated...
inhibition selectively decreased the number of Tregs in vivo. Treg/Tconv balance evaluation provided us with a potential strategy to modulate the ratio, compared with Tconvs. This selective decrease in Treg proliferation both in naïve and tumor-bearing mice. We found that this translated into a therapeutic effect in which the in vivo treatment of tumor-bearing mice with PI3K–Akt pathway inhibitors displayed a significant antitumor therapeutic efficacy. The inhibition of tumor growth was more profound when Akt and PI3K inhibitors were combined with an antitumor vaccine. Analysis of the TME revealed an enhanced antitumor immune response to the vaccine when combined with PI3K–Akt pathway inhibitors. On their own, or in combination with vaccines, the antitumor therapeutic effects of Akt and PI3K inhibitors were found to be Treg dependent as they could be reversed by Treg reconstitution.

It is interesting to point out the apparent difference between the inhibition of Akt and PI3K and the downstream inhibition of mTOR. mTOR inhibition with rapamycin has been shown to exhibit the opposite effect as it supports the proliferation and survival of Tregs (data not shown; refs. 40–43) and is therefore used as an immunosuppressant. The opposing effects of PI3K–Akt versus mTOR inhibition on Tregs may be explained by a feedback loop in which mTOR inhibition results in PI3K-dependent Akt activation, which, in turn, sustains signaling through mTOR (44).

It is also important to highlight the opposite role that the PI3K–Akt pathway plays in the de novo differentiation of mouse Tregs. Active Akt signaling has been shown to be a potent suppressor of differentiation of human (45) and mouse CD4 Tregs (46).

Figure 6. PI3K–Akt inhibition reduces tumor growth in a Treg-dependent manner. Mice were prophylactically treated with wortmannin (WM), triciribine (TCN), or DMSO before s.c. CT26 tumor inoculation. Ex vivo–cultured Tregs were infused in wortmannin-treated mice on day 4 after tumor inoculation. Tumor growth was monitored and the Foxp3 inﬁltrate was then assessed on days 20 and 24. A, prophylactic treatment of the mice with triciribine for 1 week before s.c. tumor inoculation resulted in a significant tumor growth inhibition (P < 0.0001). B, prophylactic treatment of the mice with wortmannin for 1 week before s.c. tumor inoculation resulted in a significant tumor growth inhibition wortmannin (P < 0.0001). This effect was reversed when Tregs were reconstituted by ex vivo–cultured Treg infusion on day 4. C, analysis of Foxp3+ T-cell infiltration into CT26 tumor (days 20 and 24) elucidated a significant decrease in Treg numbers per million tumor cells after wortmannin treatment (P < 0.01), which was restored after the infusion of ex vivo–grown Tregs. *P < 0.01; **P < 0.001; ***P < 0.0001.

the impact of Akt and PI3K inhibition on Tregs and TconvS to discern any selective effect that inhibition of the PI3K–Akt pathway might have on these CD4 T-cell subsets.

We demonstrated that several molecular inhibitors that target PI3K and its downstream effector, Akt, selectively inhibit the in vitro proliferation of human and murine Tregs when compared with TconvS. This selective decrease in Treg proliferation provided us with a potential strategy to modulate the Treg/Tconv balance in vivo. We found that Akt and PI3K inhibition selectively decreased the number of Tregs in vivo both in naïve and tumor-bearing mice. We found that this
potential suggests that PEK–Akt inhibitors could be exploited in the clinic as immune modulators in cancer therapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
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Selective Inhibition of Regulatory T Cells by Targeting the PI3K–Akt Pathway

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