Loss of PTEN Promotes Resistance to T Cell–Mediated Immunotherapy

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ABSTRACT

T cell-mediated immunotherapies are promising cancer treatments. However, most patients still fail to respond to these therapies. The molecular determinants of immune resistance are poorly understood. We show that loss of PTEN in tumor cells in preclinical models of melanoma inhibits T cell-mediated tumor killing and decreases T-cell trafficking into tumors. In patients, PTEN loss correlates with decreased T-cell infiltration at tumor sites, reduced likelihood of successful T-cell expansion from resected tumors, and inferior outcomes with PD-1 inhibitor therapy. PTEN loss in tumor cells increased the expression of immunosuppressive cytokines, resulting in decreased T-cell infiltration in tumors, and inhibited autophagy, which decreased T cell-mediated cell death. Treatment with a selective PI3Kβ inhibitor improved the efficacy of both anti–PD-1 and anti–CTLA-4 antibodies in murine models. Together, these findings demonstrate that PTEN loss promotes immune resistance and support the rationale to explore combinations of immunotherapies and PI3K–AKT pathway inhibitors.

SIGNIFICANCE: This study adds to the growing evidence that oncogenic pathways in tumors can promote resistance to the antitumor immune response. As PTEN loss and PI3K–AKT pathway activation occur in multiple tumor types, the results support the rationale to further evaluate combinatorial strategies targeting the PI3K–AKT pathway to increase the efficacy of immunotherapy. Cancer Discov; 6(2); 202–16. © 2015 AACR.

See related commentary by Rizvi and Chan, p. 128.

INTRODUCTION

T cells play an important role in cancer immunosurveillance and tumor destruction, and therapies that enhance antitumor T-cell responses have achieved encouraging clinical results. PD-1 checkpoint blockade and adoptive T-cell therapy (ACT) can induce objective responses in 33% to 48% of patients with metastatic melanoma, many of which are durable (1–3). However, the majority of patients still fail to respond to T cell-mediated immunotherapy and little is known about why such treatment failures occur. Understanding the pathways that cause resistance would improve the clinical application of immunotherapies through improved patient selection. Such understanding may also identify rational, more effective therapeutic combinations. Our group and others have shown that oncogenic signaling by BRAF, which is mutated in ~50% of melanomas, modulates the immune microenvironment to perturb T cell-mediated antitumor responses. Mutant BRAF increases the expression of IL1α and IL1β by tumor cells, which increases the expression of PD-L1 and PD-L2 in tumor-associated fibroblasts and suppresses the function of tumor-infiltrating T cells (TIL; ref. 4). BRAF inhibition increases the expression of melanocytic antigens (5) and inhibits VEGF production by melanoma cells, thereby enhancing trafficking of tumor-reactive T cells to tumors (6). Clinical trials evaluating the safety and efficacy of BRAF inhibitors in combination with immunotherapies are currently under way. In addition, activation of the β-catenin pathway, another oncogenic pathway, was found to be associated with poor tumor infiltration of T cells, reported in a recent publication (7). Together, these results indicate that the impact of tumor-intrinsic pathways is not always confined to tumor cells and can be extended to antitumor immune responses, especially T-cell responses.

The PI3K pathway plays a critical role in cancer by regulating several critical cellular processes, including proliferation and survival. One of the most common ways that this pathway is activated in cancer is by loss of expression of the tumor suppressor PTEN, which is a lipid phosphatase that dampens the activity of PI3K signaling. Loss of PTEN corresponds with increased activation of the PI3K–AKT pathway in multiple tumor types (8). Loss of PTEN occurs in up to...
30% of melanomas, frequently in tumors with a concurrent activating BRAF mutation (9). Although expression of mutant BRAF alone fails to transform melanocytes, invasive and spontaneously metastatic lesions develop when this is complemented by loss of PTEN in mouse models (10, 11). Loss of PTEN in patients with melanoma with BRAF mutations is associated with worse outcomes in stage III patients, and in stage IV patients treated with FDA-approved BRAF inhibitors (12, 13). Several studies have demonstrated that melanoma cell lines with loss of PTEN can be growth arrested by BRAF and MEK inhibitors but that they are resistant to apoptosis induction (14, 15). These studies support that PTEN loss identifies a distinct, clinically significant subset of melanomas.

In this study, we evaluated the impact of loss of PTEN on T cell–mediated antitumor responses. Our studies in preclinical models and clinical specimens demonstrate that loss of PTEN promotes resistance to immunotherapy in melanoma. Our findings provide new insights into the role of PTEN in cancer and identify new strategies to increase the efficacy of immunotherapy in patients.

RESULTS

Silencing PTEN Expression in Melanoma Reduces T Cell–Mediated Tumor Killing In Vitro and In Vivo

Because PTEN loss is most common in patients with melanoma with concurrent BRAF mutations, we silenced PTEN expression in established BRAF-mutant human melanoma cell lines and evaluated antitumor response to T cell–mediated immunotherapy using our previously described melanoma model (Supplementary Fig. S1A; ref. 6). Briefly, the melanoma tumor antigen gp100 and the murine MHC class I molecule H-2D<sup>+</sup> were ectopically and constitutively expressed in the human melanoma cell line A375. Gp100- and H-2D<sup>+</sup>-expressing A375 cells (A375/GH) can be recognized by T cells derived from PMEL-1 mice in an MHC class I-dependent manner. This model enables identification of immune resistance mechanisms that are independent of tumor antigen and/ or MHC loss in tumors.

Western blotting confirmed decreased PTEN and increased phosphorylated (activated) AKT (pAKT) expression in A375 melanoma cells stably transfected with shRNA targeting PTEN (A375/GH/shPTEN; Fig. 1A). Silencing of PTEN significantly reduced the percentage of lysed (cleaved caspase-3<sup>+</sup>) tumor cells when the melanoma cells were cocultured with the tumor-reactive PMEL-1 T cells in vitro (Fig. 1B). To evaluate the in vivo effects of PTEN loss on T cell–mediated antitumor activity, we used an established ACT murine model (Fig. 1C; ref. 6). PTEN loss significantly reduced the accumulation of transferred tumor-reactive T cells in A375 melanoma tumors in vivo (Fig. 1D and E). The adoptively transferred PMEL-1 T cells showed significantly reduced therapeutic activity in mice bearing PTEN-silenced tumors when compared to mice bearing PTEN-expressing tumors (Fig. 1F and G). Similarly, impaired T cell–mediated antitumor activity against PTEN-silenced tumors was also observed in the context of concurrent treatment with a selective BRAF inhibitor (Supplementary Fig. S1B–S1F). Collectively, our in vitro and in vivo studies indicate that PTEN loss can cause resistance to T cell–mediated antitumor immune responses.

PTEN Loss Correlates with Decreased Numbers and Impaired Function of Tumor-Infiltrating T Cells, and Inferior Outcomes with Anti–PD-1 in Patients with Melanoma

To determine the clinical relevance of these findings, we analyzed PTEN expression in samples from patients with melanoma. Tumors with less than 10% of cells with PTEN expression by IHC staining were classified as PTEN absent, as our previous studies demonstrated that this correlates with increased activation of the PI3K–AKT pathway (12); all other tumors were categorized as PTEN present (Fig. 2A). Analysis of a cohort of 39 patients with metastatic melanoma treated with FDA-approved anti–PD-1 antibodies (pembrolizumab and nivolumab) demonstrated that patients with PTEN-present tumors achieved significantly greater reduction of tumor size than patients with PTEN-absent tumors (P = 0.029; Fig. 2B and C). No significant differences in gender, age, stage of disease, target tumor size, or serum lactate dehydrogenase were detected between patients with PTEN-present tumors and PTEN-absent tumors (Supplementary Table S1).

We next attempted to analyze if PTEN status correlated with clinical outcomes with TIL therapy. However, we observed that the overwhelming majority of patients treated with TIL exhibited PTEN expression (44/48) in their harvested tumors, thus precluding the ability to make meaningful conclusions about the impact of PTEN loss on treatment outcomes. Analysis of tumor harvests for TIL expansion, which must be achieved successfully in order for TIL therapy to be feasible (16), showed that 26% of melanomas that did not yield successful TIL growth were PTEN-absent tumors, which was more frequent than was observed in tumors that yielded successful TIL growth (11%, P = 0.04; Fig. 2D). Thus, it is likely that the lower success rate for TIL growth from melanomas with PTEN loss leads to the exclusion of PTEN-absent patients from the TIL treatment cohort. However, other factors, such as the poor prognosis of patients with metastatic melanoma with PTEN loss (12), may also contribute to this exclusion.

Consistent with the observed association of unsuccessful TIL expansion with PTEN loss, analysis of a cohort of 135 resected stage IIIb/C melanoma regional metastases (12) found that melanomas with PTEN loss had significantly lower CD8<sup>+</sup> T-cell tumor infiltration than tumors with PTEN expression (P < 0.001; Fig. 2E; Supplementary Fig. S2A). CD8<sup>+</sup> T-cell infiltration did not correlate significantly with BRAF/NRAS mutation status, and both BRAF-mutant and BRAF/NRAS wild-type (WT) melanomas with loss of PTEN had significantly less CD8<sup>+</sup> T-cell infiltration than melanomas with PTEN expression (Supplementary Fig. S2B and S2C). Notably, in the small number of tumors (6%) that demonstrated a heterogeneous, “clonal-like” pattern of PTEN expression (Fig. 2A), more CD8<sup>+</sup> T cells were observed infiltrating regions with PTEN expression compared to adjacent regions with PTEN loss (Fig. 2F).

We further evaluated the relationship between PTEN expression and immune infiltrates using the publicly available melanoma Cancer Genome Atlas (TCGA) dataset. As we could not perform IHC, tumors were categorized based on
**Figure 1.** Reduced T cell–mediated antitumor activity against PTEN-silenced melanoma cells. A, PTEN expression and AKT activation in A375/GH cells with and without PTEN silencing. Two PTEN-silenced tumor cell lines (17 and 60) were independently produced by two shRNAs targeting PTEN (shPTEN). Tumor cells expression scrambled shRNA (shNS) served as control (PTEN-WT Tu). B, T cell–induced apoptosis rate of melanoma tumor cells with and without PTEN silencing. Two PTEN-silenced tumor cell lines (17 and 60) were independently produced by two shRNAs targeting PTEN (shPTEN) or control (shNS) to evaluate effector and target cells (E:T). The cleavage of caspase-3 in tumor cells was determined by flow cytometry. C, experimental setup of the murine ACT protocol to evaluate in vivo T cell-mediated antitumor activity. D, T-cell infiltration of melanoma tumors with and without PTEN silencing in vivo. Luciferase-expressing PMEL-1 T cells were transferred into B6 nude mice bearing A375/GH tumor with or without PTEN silencing. E, tumor growth and F, Kaplan-Meier survival curves for tumor-bearing B6 nude mice treated with adoptive transfer of PMEL-1 T cells. Similar results were obtained in repeated experiments. In D–G, three to five mice per group were used. *, P < 0.05.

**PTEN** gene copy number (CN) as gene deletion appears to be one of the most common mechanisms of PTEN loss in melanoma (17). As expected, increased expression of pAKT was observed in tumors with low (≤0.4) PTEN CN, although we cannot exclude the potential contribution of nontumor cells to this result (Fig. 3A). In contrast, the expression of LCK, a protein expressed predominantly by T cells, and the transcripts of the T-cell effector molecules IFNγ and granzyme B were significantly decreased in tumors with low PTEN CN (Fig. 3A and B). The immune cytolytic activity score defined in a recent pan-TCGA study (18) was also significantly reduced in melanomas with low PTEN CN (Supplementary Fig. S2D). Tumors with low PTEN CN also had lower histologically determined lymphocyte infiltration scores (Lscores; Fig. 3C; ref. 19). Lscores were also inversely related to expression of pAKT (Supplementary Fig. S2E).

A recent analysis of the melanoma TCGA demonstrated that activating mutations in *CTNNB1* and increased activation of the β-catenin pathway are enriched in tumors with a non-T cell–inflamed pattern of gene expression compared to tumors with a T cell–inflamed signature (7). Similarly, we observed that the frequency of deletions and loss-of-function mutations in the *PTEN* gene was higher in the non-T cell–inflamed tumors than in the T cell–inflamed melanomas (19% vs. 6%, P < 0.01; Fig. 3D). The alterations in *PTEN* and the β-catenin pathway were largely nonoverlapping, as only 2 tumors (2%) in the non-T cell–inflamed cohort had alterations in both. The association of activation of β-catenin and...
loss of PTEN was tested by a $\chi^2$ test ($\chi^2 = 0.81$ and $P = 0.37$, indicating that the tested events are not dependent). These data support that tumor-intrinsic activation of $\beta$-catenin and loss of PTEN represent two independent events associated with T-cell exclusion from the tumor microenvironment. In summary, our analyses of independent cohorts of melanoma clinical samples are consistent with the preclinical observation that loss of PTEN in melanoma reduces the recruitment and function of T cells in tumors and promotes immune resistance.

**Effects of PTEN Loss on Immune Modulators Expressed on and Produced by Tumor Cells**

To interrogate the underlying immune-suppressive mechanisms associated with PTEN loss, we first evaluated PD-L1 expression (20, 21). Silencing PTEN expression in both A375 and WM35 cell lines did not significantly increase the surface expression level of PD-L1 in vitro (Fig. 4A), nor was PD-L1 mRNA expression affected in day 14 PTEN-silenced xenograft tumors in vivo (Fig. 4B). We also observed similar patterns of PD-L1 expression between the PTEN-negative and PTEN-positive tumor areas from clinical samples with heterogeneous PTEN expression (Fig. 4C), and PD-L1 expression did not correlate with PTEN status in the cohort of stage IIIB/C melanoma patients. These findings, which are consistent with results in a large panel of human melanoma cell lines (22), suggest that PD-L1 is not the primary mechanism of the suppressed antitumor immune response induced by PTEN loss. In addition, PTEN loss did not correlate with changes in the expression of MHC class I molecules in the cohort of stage IIIB/C melanomas (Supplementary Fig. S2F).

To identify PTEN-regulated immunomodulatory genes, PTEN-silenced or control A375/GH xenografts were harvested in vivo to determine the mRNA and protein expression of chemokines and cytokines involved in the recruitment and/or function of TILs (Supplementary Table S2). Differentially expressed genes are shown in Fig. 5A. Both RT-PCR analysis and Luminox assays confirmed that loss of PTEN significantly upregulates CCL2 and VEGF (Fig. 5A and B). VEGF IHC staining confirmed that melanoma clinical samples with heterogeneous PTEN expression demonstrated increased VEGF in regions with loss of PTEN (Supplementary Fig. S3). Preclinically, functional testing confirmed that anti-VEGF-blocking antibody enhanced tumor infiltration and...
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A box-and-whisker plot was used to demonstrate the differences in expression levels of indicated genes or proteins between these two groups. A, comparison of the intensity of phosphorylated AKT and LCK in melanomas obtained from patients with different PTEN CNs according to RPPA. B, the mRNA expression levels for genes encoding IFNγ and granzyme B in tumor samples obtained from melanoma patients with different PTEN CNs. The comparison of lymphocytic infiltration score (Lscore), as determined by pathological review, between groups of patients with different PTEN CNs. The P values of the comparisons were determined by unpaired t test. D, frequencies of genetic alterations in the β-catenin pathway and PTEN between T cell–inflamed and non-T cell–inflamed tumors. Metastatic melanomas from TCGA were first catalogued based on T-cell infiltration and subsequently based on activating mutations in β-catenin itself (CTNNB1) or loss-of-function (LOF) mutations in negative regulators of the β-catenin pathway (APC, AXIN1, and AXIN2), and PTEN deletion or PTEN mutations. Non-T cell–inflamed tumors have an increased frequency of PTEN alterations compared to the T cell–inflamed tumors (P < 0.01 by Fisher exact test).

Figure 3. Reduced number and impaired effector functions of TILs in tumors with PTEN deletion or loss-of-function mutations in PTEN. Cutaneous melanoma patients whose information was included in TCGA were stratified based on the PTEN CN (cutoff, ≤ −0.4, which was chosen in order to maximize the difference in the level of activity of the AKT pathway). A box-and-whisker plot was used to demonstrate the differences in expression levels of indicated genes or proteins between these two groups. A, comparison of the intensity of phosphorylated AKT and LCK in melanomas obtained from patients with different PTEN CNs according to RPPA. B, the mRNA expression levels for genes encoding IFNγ and granzyme B in tumor samples obtained from melanoma patients with different PTEN CNs. C, comparison of lymphocytic infiltration score (Lscore), as determined by pathological review, between groups of patients with different PTEN CNs. The P values of the comparisons were determined by unpaired t test. D, frequencies of genetic alterations in the β-catenin pathway and PTEN between T cell–inflamed and non-T cell–inflamed tumors. Metastatic melanomas from TCGA were first catalogued based on T-cell infiltration and subsequently based on activating mutations in β-catenin itself (CTNNB1) or loss-of-function (LOF) mutations in negative regulators of the β-catenin pathway (APC, AXIN1, and AXIN2), and PTEN deletion or PTEN mutations. Non-T cell–inflamed tumors have an increased frequency of PTEN alterations compared to the T cell–inflamed tumors (P < 0.01 by Fisher exact test).

improved antitumor activity of transferred tumor-reactive T cells against A375/GH/shPTEN xenografts (Fig. 5C and D). These results and previously published studies (23, 24) suggest that loss of PTEN in melanomas promotes resistance to immune infiltration of tumors through the production of inhibitory cytokines.

We extended our investigation of the factors mediating the immunosuppressive effects of PTEN loss by analyzing the gene expression of 609 inflammation-related genes in tumors from 47 patients with metastatic melanoma, which include 10 samples with absent PTEN (Supplementary Fig. S4A). The vast majority of analyzed genes (504/609) exhibited reduced expression in the tumors with PTEN loss, suggesting a lack of inflammatory cells in the tumor microenvironment. To define genes that were consistently altered, we utilized a leave-one-out logistic regression analysis (see Supplementary Information). This analysis identified TNFRSF10C, PDGFRB, TLR3, MASP2, and CLU as genes significantly correlated with PTEN in at least 8 distinct models, and all 5 genes correlated positively with PTEN expression (Supplementary Fig. S4B).
Figure 4. PD-L1 expression is not associated with PTEN expression status in melanomas. A, the PD-L1 surface expression levels in melanoma cells with and without PTEN silencing. A375 and WM35 tumor cells were transduced with control shRNA or PTEN-specific shRNA. Transduced tumor cells were stained with anti–PD-L1 to evaluate the surface expression of PD-L1. B, the in vivo PD-L1 expression in melanoma tumor tissues with and without PTEN silencing. B6 nude mice were challenged with A375/GH/shPTEN or A375/GH/shNS tumor cells. Tumor samples were collected from mice bearing 14-day established tumors. The PD-L1 mRNA expression levels in tumor samples were determined using real-time PCR. C, examples of IHC for PD-L1 in tumors with clonal PTEN expression. D, the PD-L1 surface expression levels in tumor samples from patients with stage IIIB/C melanoma. Tumor samples were placed in PTEN-absent and PTEN-present groups, and the expression of PD-L1 on the tumor surface was determined by IHC.

The area under the ROC curve (AUC) obtained by this classifier of the 5 genes was 91.6% (Supplementary Fig. S4C), indicating a strong performance in discriminating between PTEN-replete and PTEN-loss melanomas.

Reduced Autophagic Activity in PTEN-Loss Melanoma Promotes the Resistance to T Cell–Induced Tumor Apoptosis

Because infiltrating nontransformed cells in both clinical specimens and xenografts could potentially confound our ability to identify tumor-specific gene expression changes mediated by PTEN, we performed a microarray-based gene expression analysis in human melanoma cell lines with PTEN silencing and compared the results to the analyses described above. The expression levels of the 5 genes identified by the leave-one-out logistic regression analysis in tumor tissue were comparable in tumor cells with and without PTEN silencing, making it likely that these genes were expressed in infiltrating cells in the tumor tissues. However, this approach identified ATG16L as a gene upregulated by PTEN in both melanoma cell lines and clinical specimens (Supplementary Fig. S4A). The ~800-kDa protein complex formed by ATG16L conjugated with ATG12 and ATG5 is required for efficient LC3 lipidation, a critical step in autophagy. Interestingly, several genome-wide association studies suggest that ATG16L mutation is a risk factor for inflammatory bowel disease (25, 26).

We hypothesized that PTEN loss promotes resistance to T cell–mediated killing by inhibiting autophagy. Western blotting of protein from melanomas with PTEN loss demonstrated reduced LC3 lipidation, as determined by the level of LC3II (Supplementary Fig. S5A), and PTEN knockdown in human melanoma cell lines decreased the expression of both LC3I and LC3II (Fig. 6A). We then performed functional testing in three patient-derived melanoma cell lines that undergo apoptosis when exposed to their autologous TILs. We perturbed the expression of genes required for activation of autophagy (Supplementary Table S3) in the tumor cells and exposed them to autologous TILs. A comboscore was calculated based...
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B6 nude mice were challenged with A375/GH/shPTEN. Seven days after tumor challenge, luciferase-expressing CD8+ T cells were transferred into tumor-bearing mice. Bioluminescence imaging was performed to evaluate tumor trafficking of transferred T cells in three to five mice per group were used.

Figure 5. Critical role of VEGF in immune resistance associated with loss of PTEN. A and B, B6 nude mice were challenged with A375/GH/shPTEN or A375/GH/shNS melanoma cells, and tumor samples were collected from 14-day tumor-bearing mice. A, transcript expression levels for cytokine and chemokine genes in tumor samples as determined using quantitative RT-PCR. B, protein expression of cytokines and chemokines in tumor samples as determined by Luminex analyses. C and D, B6 nude mice were challenged with A375/GH/shPTEN. Seven days after tumor challenge, luciferase-expressing PMEL-1 T cells were transferred into tumor-bearing mice. Bioluminescence imaging was performed to evaluate tumor trafficking of transferred T cells in treated mice. Tumor growth (C) and T-cell infiltration (D) of PTEN-silenced tumors in mice treated with ACT and/or anti-VEGF antibody. Representative imaging figures and quantification of the intensity of luciferase at tumor site from all tested mice are shown. Quantification was expressed as the average of photon flux within ROI. In C and D, three to five mice per group were used. 

The results support that PTEN loss can protect tumor cells from T-cell killing through an autophagy-dependent mechanism.

PI3Kβ Inhibitor Improves the Activity of Checkpoint Blockade for PTEN-Loss Tumors

We hypothesized that inhibiting the PI3K pathway would improve the effectiveness of immunotherapy. Although inhibition of some targets in this pathway are critical to immune cell function and viability, the PI3Kβ isoform can regulate AKT activity in tumors with PTEN loss, yet it is dispensable in the activation of the TCR signaling pathway (27, 28). Therefore, we tested whether selective inhibition of PI3Kβ would increase the efficacy of immunotherapy in melanomas with PTEN loss.

In vitro treatment with a selective PI3Kβ small-molecule inhibitor, GSK2636771, reduced the activation of the AKT pathway and moderately (~20%) inhibited the growth of three human melanoma cells with loss of PTEN (Supplementary Fig. S6A and S6B). To examine the role of the PI3Kβ inhibitor in T-cell-induced apoptosis, the three PTEN-null human melanoma cell lines were engineered to express gp100 and murine H2-D6, and then tested for caspase-3 cleavage after coinubation with the PI3Kβ inhibitor in the presence of PMEL-1 T cells. Treatment with the PI3Kβ inhibitor improved the T-cell–induced tumor killing of all three PTEN-null melanoma cell lines (Supplementary Fig. S6C).
PTEN expression regulates autophagy in tumor cells, reducing T cell–mediated killing. A, expression of LC3 I and LC3 II in BRAF-mutant melanoma cell lines with and without PTEN silencing. The expression levels of LC3 I and LC3 II in protein lysates from A375 and WM35 tumor cells were determined by Western blotting analysis. B, perturbing the expression of autophagy-related genes changes the sensitivity of melanoma to apoptosis induced by tumor-reactive T cells. A patient-derived melanoma cell line, Mel2400, was transduced with lentiviral vectors encoding the open reading frames (ORF) of autophagy-related genes. Virally transduced tumor cells were cocultured with paired autologous T cells for 3 hours. The percentage of killed (cleaved caspase-3 or shRNAs of autophagy-related genes. Virally transduced tumor cells were cocultured with paired autologous T cells for 3 hours. The percentage of apoptotic tumor cells was evaluated by the cleaved caspase-3 assay. Results are representative of data generated in two independent experiments.

We then compared the effects of the PI3Kβ inhibitor and a pan-PI3K inhibitor (BKM120) on the effector function of antigen-specific T cells using an established murine vaccine model (29). Brieﬂy, C57BL/6 mice were adoptively transferred with splenocytes from PMEL-1 mice followed by hgp100−25–33 peptide vaccination on day 0 and day 30. Vaccinated mice were treated with PI3K inhibitors daily for 5 days. Treatment with the pan-PI3K BKM120 inhibitor reduced whole-blood cell counts and inhibited the proliferation of gp100−specific T cells upon initial immunization, whereas the PI3Kβ inhibitor did not affect either parameter (Fig. 7A; Supplementary Fig. S6D). Importantly, the PI3Kβ inhibitor did not affect the percentage of antigen-speciﬁc T cells in mice receiving a booster vaccine (Fig. 7A). These results support that the generation of memory T cells remains intact with PI3Kβ inhibitor treatment.

The recent establishment of Tyr:Crep;BrafV600E/∗;Ptenﬂox mice (BP mice) in a C57BL/6 background, which spontaneously develop Braf-mutant, Pten-null melanomas after the induction of Cre expression, provides a valuable model to evaluate the therapeutic effects of cancer treatments in melanoma with PTEN loss (11). Treatment of BP mice in vivo with the PI3Kβ inhibitor reduced pAKT expression in the tumors, whereas treatment with the pan-PI3K inhibitor BKM120 at the dose that was suppressive to T cells did not (Supplementary Fig. S6E). Reverse phase protein array (RPMA) analysis conﬁrmed that PI3Kβ inhibitor treatment decreased the phosphorylation of AKT and other PI3K pathway activation markers (e.g., pmTOR; Fig. 7B). To test the antitumor activity of checkpoint blockade combined with the PI3Kβ inhibitor, BP mice bearing measurable melanoma...
lesions were randomly treated with solvent/antibody control, PI3Kβ inhibitor, anti–PD-1 antibody, or the combination of PI3Kβ inhibitor and anti–PD-1. Treatment with each single agent had minimal effect, but combined treatment with the PI3Kβ inhibitor and anti–PD-1 significantly improved tumor growth inhibition and survival of the mice (Fig. 7C and D). PI3Kβ inhibition also demonstrated synergy in combination with anti–CTLA-4 in vivo in the same model (Supplementary Fig. S7A–S7C). Testing was also performed using a transplanted tumor model in which BP cells (from an established murine BRAF-mutant and PTEN-loss cell line) were implanted subcutaneously in C57BL/6 mice (30). Tumor-bearing mice were treated with anti–PD-1 and/or the PI3Kβ inhibitor (Supplementary Fig. S7D). Similar to the results in the spontaneous tumor model, the PI3Kβ inhibitor enhanced the efficacy of immunotherapy (Supplementary Fig. S7E). The combination of the PI3Kβ inhibitor and checkpoint inhibition also significantly increased the number of infiltrating CD4+ and CD8+ T cells in both the spontaneous tumor model and the transplanted tumor model (Fig. 7E). Taken together, these data support that PI3Kβ inhibition can improve the efficacy of immunotherapy in melanomas with PTEN loss.

**DISCUSSION**

T cell-mediated immunotherapies, including ACT and checkpoint blockade, have demonstrated that durable disease eradication and survival is achievable even in patients with...
the most advanced stages of cancer. However, the majority of cancer patients currently fail to achieve such clinical benefit. Although the development of novel immunologic strategies to enhance the function of tumor-reactive T cells is an important and promising approach, there is also a growing appreciation of the significance of tumor-associated factors as key and potentially targetable determinants of the efficacy of immunotherapy.

Melanoma was one of the first solid tumors in which immunotherapies were shown to have clinical benefit. High-dose IL2 was approved for the treatment of patients with metastatic melanoma in 1998, based primarily on the achievement of long-term (>10 years) survival in ~5% of patients (31). In recent years, three immune checkpoint inhibitors (ipilimumab, pembrolizumab, and nivolumab) have gained regulatory approval for this disease, with higher durable response rates (32). Clinical responses have also been achieved in approximately 50% of patients receiving lymphodepletion followed by ACT (3). In parallel to these advances in immunotherapy, there has been a tremendous increase in the understanding of the molecular pathogenesis of melanoma. We and others have demonstrated that oncogenic mutations in \textit{BRAF}, which activate the RAS–RAF–MAPK pathway, regulate several factors critical to the antitumor immune response, including tumor antigen expression and the production of immunosuppressive cytokines (4–6). A number of clinical trials have been initiated for testing the safety and efficacy of combining RAS–RAF–MAPK pathway inhibitors and immunotherapies. Although \textit{BRAF} mutations are highly prevalent in melanoma, they are insufficient to transform melanocytes by themselves. Experiments in both human melanocytes and murine models demonstrated that concurrent loss of PTEN, which activates the PI3K–AKT pathway, complements \textit{BRAF} mutations to produce invasive and metastatic melanomas (10, 11). Additional studies have demonstrated that loss of PTEN in melanoma promotes activation of the PI3K–AKT pathway, tumor invasiveness, and resistance to cell killing by \textit{BRAF} and MEK inhibitors. In this study, we now provide new evidence that loss of PTEN also contributes to immune resistance in this disease.

We have shown for the first time that melanomas with loss of PTEN protein expression have decreased infiltration by CD8+ T cells in both \textit{BRAF}-mutant and \textit{BRAF/ NRAS}-WT melanomas. In addition to the analysis of samples collected from patients treated at our institution, our TCGA analyses reveal that low PTEN CN is associated with reduced LCK expression, Lscore, and cytolytic activity in melanomas, findings consistent with decreased immune infiltration with PTEN loss. In addition, \textit{PTEN} deletions and loss of function mutations are also significantly enriched in the non-T cell–inflamed tumors in the melanoma TCGA samples. Notably, our analysis of the melanoma TCGA supports that loss of PTEN in melanomas is largely nonoverlapping with \textit{β}-catenin pathway alterations, which have recently been shown to promote immunosuppressive effects (7). These results suggest that multiple distinct genetic events can give rise to immune exclusion. However, even oncogenic activation of the \textit{β}-catenin pathway and PI3K pathway does not account for all of the tumors with the non-T cell–inflamed phenotype. Additional work will be necessary to identify additional molecular events that may explain this phenotype in the remaining patients.

In contrast to some other reports (20, 21, 33), our analyses of cell lines, \textit{in vitro} models, and clinical specimens did not detect increased PD-L1 expression in melanomas with loss of PTEN. This discrepancy may be caused by the difference in tumor types and/or cell lines selected in these studies. However, our results are consistent with a recent analysis of 51 melanoma cell lines (22). While the observed increase in PD-L1 expression with PTEN loss was primarily observed \textit{in vitro}, the \textit{in vivo} expression of PD-L1 in tumors is dynamic and regulated by many factors, such as IFNγ from lymphocytes (34). Taken together, it does not appear that the immunosuppressive effect of PTEN loss is explained by upregulation of the expression of PD-L1 in melanomas.

Similar to oncogenic \textit{BRAF}, PTEN loss induced the expression of a number of immunosuppressive cytokines, particularly VEGF, which was validated in clinical samples. VEGF was originally identified as a key tumor-associated factor that induces changes in blood vessel permeability and architecture (35). Besides its well-documented role in angiogenesis, VEGF can contribute to the immunosuppressive tumor microenvironment by recruiting suppressive immune cells, such as immature dendritic cells, myeloid-derived suppressor cells (MDSC) and regulatory T cells (36). Our studies reported here, and an independent study in B16 murine tumors, have shown that blocking VEGF can result in increased trafficking of tumor-reactive T cells to tumors \textit{in vivo} (23). These results support VEGF inhibition as a rational combinatorial strategy in melanoma and suggest that ongoing clinical trials with VEGF inhibitors in this disease should include the analysis of effects on T-cell trafficking, particularly in melanomas with loss of PTEN.

In the modified xenograft model we used for mechanistic studies, expression of the MHC class I (H-2Dβ) is under the control of a constitutively activated promoter, and tumor-reactive T cells are \textit{in vitro} activated by TCR stimulation. This model is therefore inappropriate to evaluate the effect of PTEN loss on the initial priming of T cells and regulation of MHC class I expression. Although we cannot fully exclude a role for those mechanisms, in melanoma patient samples with heterogeneous PTEN expression we observed differing T-cell infiltration in regions with and without PTEN expression. Given that the initial priming of TILs in PTEN-positive areas should be comparable with TILs in PTEN-negative areas within these patients, it is unlikely that antigen priming is the exclusive mechanism by which PTEN regulates the antitumor immune response. Moreover, our analysis of additional clinical samples using Nanostring technology and IHC indicates that the expression of MHC class I is not affected by PTEN loss. Thus, effects on MHC class I do not appear to be essential for immune resistance associated with PTEN loss. Also, due to the lack of MDSC in the xenograft model, further investigations are required to determine their role in PTEN loss–induced immune resistance in melanoma, as was alluded to in a recent report using a mouse model of prostate cancer (37).

Interrogation of the molecular consequences of PTEN loss implicates inhibition of autophagy as a contributor to the observed resistance to tumor cell killing by T cells. The role
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and therapeutic potential of autophagy in cancer is complicated (38), as it may induce or inhibit cell death of cancer cells in a context-dependent manner. Although it has previously been reported that autophagy induced by chemotherapeutic agents or radiation can promote antitumor immune responses (39, 40), the role of autophagy regulated by oncogenic pathways in T cell–mediated antitumor responses is not well characterized. The observed reduction in autophagy in melanoma cell lines with loss of PTEN is consistent with previous studies (41). Notably, we observed that treatment with the autophagy inhibitor hydroxychloroquine reduced T cell–mediated tumor killing. As inhibition of autophagy is being tested clinically in multiple diseases, our results support the importance of evaluating the immune effects observed in those trials. Additional studies are also needed, and are ongoing, to further delineate the ways in which autophagy regulates the antitumor immune response.

Our findings also have significant implication for immunotherapies currently being used to treat patients with metastatic melanoma. In addition to observing a decrease in T-cell infiltration, we observed that loss of PTEN correlates with significantly lower likelihood of successful TIL generation from melanomas harvested for therapeutic intent. It is likely that this is due to PTEN loss–mediated inhibition of lymphocytic infiltration of tumors. However, we cannot exclude the possibility of other functional causes for this observation. Notably, loss of PTEN promoted resistance to direct T cell–mediated tumor killing in vitro, an effect that cannot be attributed to lymphocyte trafficking. Although these findings have significant implications for ACT, such therapies are currently available at only a limited number of cancer centers with the ability to generate and administer TILs. In contrast, both ipilimumab and PD-1 antibodies (pembrolizumab and nivolumab) are approved and widely used in patients with metastatic melanoma. We report here the novel finding that patients with metastatic melanoma with loss of PTEN expression are less likely to respond to treatment with PD-1 blocking antibodies. In addition, we have shown in preclinical models that treatment with a PI3Kβ inhibitor, which had minimal effect on melanoma growth in vitro or in vivo as a single agent, sensitized PTEN-null melanomas to in vitro killing by T cells, increased T-cell infiltration in vivo, and significantly improved tumor control and survival achieved by both CTLA-4 and PD-1 blocking antibodies in murine tumor models. Although relatively high concentrations of the PI3K inhibitor were required to see PI3K–AKT pathway blockade, early-phase clinical testing of this class of agents has demonstrated a very favorable toxicity profile, with serum concentrations in the micromolar range achieved without significant toxicity (42). Importantly, treatment with the PI3Kβ inhibitor had virtually no impact on immune cell viability or function, in marked contrast to a clinically relevant pan-PI3K inhibitor. Although there is significant debate about the relative therapeutic potential of different PI3K isoforms, we believe that our data specifically support the rationale for further testing of PI3K–AKT pathway. Because the oncogenic activation of the PI3K–AKT pathway is frequent in many other cancers in which the clinical benefit of immunotherapy is being explored, such studies may lead to the identification of additional strategies to improve the efficacy of immunotherapy, and ultimately to improve the rate of durable cures in patients with cancer.

METHODS

Human Subjects and Clinical Response Evaluation

The study was conducted in accordance with the Declaration of Helsinki. All patients provided written informed consent to participate in the study. The University of Texas MD Anderson Cancer Center (MDACC) Institutional Review Board approved all of the research protocols for this investigation. Cohorts of MDACC analyzed included 128 patients with metastatic melanoma enrolled in an ACT clinical trial; 135 patients with stage IIIB/C melanoma who underwent standard-of-care lymphadenectomy (12); and patients with metastatic melanoma treated with single-agent pembrolizumab (37 cases) or nivolumab (2 cases) with available tumor samples collected at some time prior to the start of PD-1 therapy. PD-1 response was determined by calculating the maximum change from baseline in the sum of the longest diameter of each target lesion after anti–PD-1 antibody treatment. Detailed patient characteristics of the single-agent anti–PD-1 cohort are provided in Supplementary Methods.

Animals and Cell Lines

Nude B6 mice were purchased from Taconic. TyrCreER; Ptenfl/fl; Braf<tm; mmt> mice bred onto a C57BL/6 background were kindly provided by Dr. M. Rosenberg (Yale University School of Medicine). PMEL-1 TCR/Thy1.1 mice were from in-house breeding colonies. All mice were maintained in a specific pathogen-free barrier facility and handled in accordance with protocols approved by the Institutional Animal Care and Use Committee. Human melanoma cell lines Mel2338, Mel2400, Mel2549, and their autologous TILs were established from patients with metastatic melanoma enrolled in the MDACC TIL trial, as previously described (43). Human melanoma cell lines (A375, A2058, UCD354L, WM35, WM1799, and WM2664) were purchased from the ATCC or provided by Dr. J. Gershennahald (MDACC). The murine BRAF-mutant melanoma cell line BP cell was developed by Dr. J. Wargo (MDACC), as described previously (30). All cell lines were verified in 2014 or 2015 by short-tandem repeat fingerprinting or matching mutational profiles. PMEL-1 T cells used in in vitro assays and Luciferase-expressing PMEL-1 T cells used for in vivo studies were generated as previously described (44).

Lentiviral Transduction of Tumor Cells

Gp100– and murine H-2D<sup>e</sup>–expressing A375 (A375/GH) and WM35 (WM35/GH) cells were generated previously (6). Detailed information about lentiviral vectors encoding open reading frames (ORF) of autophagy-related genes and shRNAs targeting PTEN and autophagy-related genes is provided in Supplementary Methods.

IHC

The levels of PTEN, CD8, PD-L1, and VEGF<sup>+</sup> protein expression in formalin-fixed, paraffin-embedded (FFPE) melanoma samples were assessed using IHC with anti-PTEN (6H2.1; Cascade BioScience), anti-CD8
(C8/144B, Thermo Fisher Scientific), anti-PD-L1 (EPR11612[2], Abcam), anti-HLA class I (EMR8-5, MBL), and anti-VEGF (VG1; Thermo Fisher Scientific) antibodies, as described previously (45, 46) or as suggested by the manufacturers. Absence of PTEN was defined as <10% of tumor cells with any immunoreactivity in tumors with staining observed for internal positive controls (i.e., endothelial cells). The degree of CD8+ T-cell infiltration was measured in 10 independent high-power microscopic fields for each tissue sample. The percentage of CD8+ T-cell–present area within the tumor cell nest was recorded for each field. PD-L1 positivity (PD-L1+) was defined as ≥25% cell membrane staining of any intensity (47). The IHC staining for MHC class I was performed and scored as previously described (48). Patients with no viable tumor tissue or with a tumor expressing large amounts of melanin were excluded from this study after pathologists’ review.

**Gene Expression and Protein Expression Analysis**

The Nanosting technology was used to analyze mRNAs isolated from FFPE clinical specimens. Quantitative real-time PCR was carried out to evaluate the expression of mRNA isolated from tumor tissues in the preclinical studies. Three individual reactions were performed to measure cytokeines/chemokines in murine tumors. Antibodies against PTEN, phosphorylated AKT (S473 and T308), total AKT, LC3B and p62 (Cell Signaling Technology) were used for western blotting. Detailed information was provided in Supplementary Methods.

**TCGA Analysis**

Public TCGA data repositories for skin cutaneous melanoma were used as our sources of sample data (7, 19). Analyses included CN (SNP array), mRNA expression (RNA-seq), protein expression (RPPA), somatic mutations (exome sequencing), and lymphocyte score (pathology review).

**Caspase-3 Cleavage Cytotoxicity Assay**

The caspase-3 cleavage cytotoxicity assays were performed as previously described (49). A comboscore was calculated using the following formula. Any genetic alteration that can enhance the sensitivity of a tumor to T cell–mediated killing will have a **comboscore** greater than 1.

\[
\text{Comboscore} = \frac{[\% \text{Casp-3}\_\text{ORF/shRNA tumor with T cell}] - [\% \text{Casp-3}\_\text{ORF/shRNA tumor without T cell}]}{[5\% \text{cell membrane staining of any intensity with T cell}] - [5\% \text{cell membrane staining of any intensity with T cell}]} 
\]

**Animal Tumor Models**

ACT in 86 nude mice, in vivo vaccination, PLX4720 treatment, anti-VEGF-blocking antibody treatment, and in vivo Bioluminescence Imaging were performed as previously described (5, 27). GSK2636771 and BKM120 (Chemi Tek) were suspended in 1% (w/v) methylcellulose and administered to mice by daily oral gavage at a dose of 30 mg/kg and 60 mg/kg, respectively. For the spontaneous tumor model, **Tyr:CreER:Petn**<sup>lox/lox**:BrΔ<sub>VORE</sub>**<sup>+/–</sup> mice on a C57BL/6 background (6-8 weeks of age) were treated with 4-hydroxytamoxifen to induce the expression of Cre, as previously described (11). The antitumour PD-1 antibody (29F1A12, Biologent) was intraperitoneally injected on days 0, 2, and 4 at a dose of 100 μg per mouse. The relevant solvent and control rat IgG antibody (Sigma) were administered to control animals.

**Statistical Analyses**

Summary statistics (e.g., mean, SEM) of the data are reported. Assessments of differences in continuous measurements between two groups were made using two-sample t test posterior to data transformation (typically logarithmic, if necessary), or Wilcoxon rank-sum test. Differences in tumor size and T-cell numbers among several treatments were evaluated using analysis of variance (ANOVA) models. The Kaplan-Meier method and log-rank test were used to compare survival between groups. P-values of less than 0.05 were considered significant. Detailed statistical analysis for the Nanostring data was provided in Supplementary Methods. Graph generation statistical analyses were performed using the Prism software program (GraphPad Software), Tableau 8.2 software program (Tableau Software), and R software programming language (version 3.1.0).

**Disclosure of Potential Conflicts of Interest**

M.T. Tetzlaff is a consultant/advisory board member for Myriad Genetics. C. Bernatchez is a consultant/advisory board member for Lion Biotechnologies. J.A. Wargo reports receiving speakers bureau honoraria from DANA and is a consultant/advisory board member for Roche-Genentech and GSK. J.E. Gershenwald is a consultant/advisory board member for Merck. M.A. Davies reports receiving commercial research grants from GSK, Genentech, Merck, Sanofi-Aventis, Myriad, AstraZeneca, and Oncothyreon and is a consultant/advisory board member for GSK, Novartis, Genentech, and Sanofi-Aventis. No potential conflicts of interest were disclosed by other authors.

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Other (established the melanoma cell lines and tumor infiltrating lymphocyte lines from patients with melanoma used in this study): M.-A. Forget

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REFERENCES


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