PI3K Inhibition Reduces Mammary Tumor Growth and Facilitates Antitumor Immunity and Anti-PD1 Responses

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Abstract

Purpose: Metastatic breast cancers continue to elude current therapeutic strategies, including those utilizing PI3K inhibitors. Given the prominent role of PI3Kα,β in tumor growth and PI3Kδ in immune cell function, we sought to determine whether PI3K inhibition altered antimtumor immunity.

Experimental Design: The effect of PI3K inhibition on tumor growth, metastasis, and antimtumor immune response was characterized in mouse models utilizing orthotopic implants of 4T1 or PyMT mammary tumors into syngeneic or PI3Kα/β-null mice, and patient-derived breast cancer xenografts in humanized mice. Tumor-infiltrating leukocytes were characterized by IHC and FACS analysis in BKM120 (30 mg/kg, every day) or vehicle-treated mice and PI3Kα/βWT versus PI3Kα/β−/− mice. On the basis of the finding that PI3K inhibition resulted in a more inflammatory tumor leukocyte infiltrate, the therapeutic efficacy of BKM120 (30 mg/kg, every day) and anti-PD1 (100 µg, twice weekly) was evaluated in PyMT tumor-bearing mice.

Results: Our findings show that PI3K activity facilitates tumor growth and surprisingly restrains tumor immune surveillance. These activities could be partially suppressed by BKM120 or by genetic deletion of PI3Kδ in the host. The antitumor effect of PI3Kδ loss in host, but not tumor, was partially reversed by CD8+ T-cell depletion. Treatment with therapeutic doses of both BKM120 and antibody to PD-1 resulted in consistent inhibition of tumor growth compared with either agent alone.

Conclusions: PI3K inhibition slows tumor growth, enhances antitumor immunity, and heightens susceptibility to immune checkpoint inhibitors. We propose that combining PI3K inhibition with anti-PD1 may be a viable therapeutic approach for triple-negative breast cancer. Clin Cancer Res; 23(13); 3371–84. ©2016 AACR.

Introduction

One of every eight women in America will develop breast cancer in her life and in the United States alone we have over 40,000 annual deaths due to this disease (1). Breast cancers often arise due to somatic genetic mutations (2), and mutations that result in activation of pathways related to PI3K are common in these tumors. PI3Ks form an evolutionarily conserved family of lipid kinases whose members include Class I A catalytic subunits (p110α, β, δ); a Class 1B catalytic subunit (p110γ); Class I regulatory domains (p85 α, β, γ, p150, p101, p87); Class II subunits (C2α, C2β, C2γ); and a Class 3 protein (Vps34). The Class IA PI3K known as PIK3CA (p110α) plays a critical role in a wide range of carcinomas and one third of somatic gene mutations in breast cancer patient tumors occur in this kinase family (2).

Several reports have shown that breast cancer patients with activating PIK3CA mutation have a poor prognosis (3, 4). In contrast, patients with estrogen receptor- or progesterone receptor–positive tumors with PIK3CA mutation have a better long-term survival (5–10). Several PI3K inhibitors are in clinical trials for many cancers and early results point to multiple mechanisms by which breast tumors do not respond or develop resistance to PI3K inhibitors (11, 12). As a result, PI3K inhibitors are being evaluated in clinical trials in combination with AKT inhibitors, CDK4/6 inhibitors, mTOR inhibitors, HER2 inhibitors, MEK inhibitors, aromatase inhibitors, enzalutamide, tamoxifen, cisplatin, and paclitaxel, but often at the expense of considerable added toxicity (13–16). GDC-0941 [potent inhibitor of PI3Kα/β (IC50 3 nmol/L) with some activity for PI3Kδ, p85α, p85β, p101, p87] and a Class II inhibitor from Novartis (IC50 of 5 nmol/L) with IC50so of 33 and 75 nmol/L] from Genentech is in phase II clinical trials (17, 18) in combination with endocrine therapy, paclitaxel/docetaxel, BLY719, a selective PI3Kα inhibitor from Novartis (IC50 of 5 nmol/L) in combination with the CDK4/6 inhibitor LEE011 is in phase Iib/II trials in ER+ HER2– metastatic breast cancer. The Novartis compound BKM120 inhibits α, δ, β, and γ with IC50s of 10.1158/1078-0432.CCR-16-2142

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Identification of targeted therapies that activate antitumor immunity could offer a significant advance for cancer patients. PI3K inhibitors when combined with other targeted therapies have shown promise for treatment of breast and other cancers. Here we show that in addition to enhancing tumor growth and metastasis, activation of the PI3K pathway in immune cells suppresses antitumor immunity in mouse models of breast cancer. Moreover, combining pan-PI3K inhibition with immune checkpoint blockade improves the therapeutic response by targeting both the tumor and its microenvironment. Our data indicate that clinical trials combining inhibitors of PI3K with anti-PD1 should be considered for triple-negative breast cancers.

PI3K activities within cancer cells are important for tumor cell growth, proliferation, and survival, motility, and metastasis; they also play vital roles in leukocyte recruitment and activation. Maintenance of vascular integrity, and other aspects of the tumor microenvironment (TME; ref. 21) 1. These features underscore the crucial need to determine the interplay between cancer cell–intrinsic functions of PI3K and antitumor immune responses. Stromal tumor-infiltrating lymphocytes (TIL) may be an indicator for better prognosis for triple-negative breast cancer (22). One concern in using pan-PI3K inhibitors is that PI3K δ and γ are important for leukocyte activation and migration, respectively (23, 24). For this reason, studies were launched using δ- and γ-sparing PI3K inhibitors.

One strategy used by transformed cells to evade immune surveillance is to activate immune checkpoints, the immune inhibitory signals that normally restrict immune cell overactivation (25–27). Alternatively, immune cells can be manipulated ex vivo and then infused back into cancer patients to provide enhanced antitumor activity (28, 29). However, many cancers, including breast cancers, are often refractory to these interventions. Use of immunotherapy, such as PD-1 and the IgG control antibody. Antibodies for FACS analysis were from Biolegend, Inc. Antibodies for CD8 depletion were from BioXCell as were anti-PD1 and the IgG control antibody. Antibodies for IHC were as follows: FoxP3 (FJK-16s), B220 (RA3-6B2), F4/80 (BM8), MHCII (M5/114.15.2). Antibodies for CD8 depletion were from BioXCell and NSG mice with NOD/SCID/IL2γnull, NSG were obtained from the Jackson Laboratory. PI3Kδ knockout mice were from Jens Stein, University of Bern (Bern, Switzerland).

BKM120 was supplied by Novartis. The pharmacokinetics of BKM120 in mouse models has been described previously (30). Antibodies for CD8 depletion were from BioXCell as were anti-PD1 and the IgG control antibody. Antibodies for FACS analysis were from Biolegend, Inc. All antibodies are Rat host, sourced from Biolegend, Inc. Blocking Ab with TruStain FCX CD16/32 (Clone #93). CD45 (30-F11), CD19 (6D5), CD3 (17A2), CD4 (RM4-5), CD8 (53-6.7), CD25 (PC61), CD11b (M1/70), CD11c (N418), Ly6c (HK1.4), Ly6g (1A8), CD49b (DX5), F4/80 (BM8), MHCI (M5/114.15.2). Antibodies for IHC were as follows: FoxP3 (FJK-16s), eBioscience, Inc., 1:100; CD4 (4S9M9) eBioscience, Inc., 1:1,000; CD8 (4S8M5) eBioscience, Inc., 1:1,500; B220/CD45RO (RA3-6B2) BD-Phar-mingen, 1:200.

Materials and Methods

Animals

Experiments were conducted in accordance with Vanderbilt University Animal Care and Use Committee guidelines (protocol number M/13/052). Generation of patient-derived xenograft (PDX) was approved by the Institutional Review Board (IRB number 130489). C57Bl/6, BALB/c, and FVB wild-type mice were purchased from Charles River Laboratories. OT-I (C57Bl/6-Tg (TcrαTcrβ)1100Mjb/J) and OT-II (C57Bl/6-Tg (TcrβJrb) 425Cbn/J) mutant mice were purchased from Jackson laboratory. NOD/SCID/IL2 receptor γ chain null (NOD/SCID)/IL2γnull, NSG and NSG mice with β-2 microglobulin deficiency (NSGb) mice were obtained from the Jackson Laboratory. PI3Kδ knockout mice were from Jens Stein, University of Bern (Bern, Switzerland).

Implantation of breast tumor cells

MMTV-PyMT mammary tumor cells (C57Bl/6 genetic background and FVBn background) were established from spontaneous tumors arising in mice (32). 4T1 cells (BALB/c genetic background) were obtained from the ATCC. Cells were injected in the left fourth mammary fat pad of 6-to 8-week-old non-parous female mice. Numbers of cells injected varied with cell type and experimental design. PDXs were generated by implanting 1–2 mm diameter pieces of tumor tissue taken from triple-negative breast cancer patients undergoing surgery or biopsy into NSG mice obtained from Jackson Laboratories.

Translational Relevance

Identification of targeted therapies that activate antitumor immunity could offer a significant advance for cancer patients. PI3K inhibitors when combined with other targeted therapies have shown promise for treatment of breast and other cancers. Here we show that in addition to enhancing tumor growth and metastasis, activation of the PI3K pathway in immune cells suppresses antitumor immunity in mouse models of breast cancer. Moreover, combining pan-PI3K inhibition with immune checkpoint blockade improves the therapeutic response by targeting both the tumor and its microenvironment. Our data indicate that clinical trials combining inhibitors of PI3K with anti-PD1 should be considered for triple-negative breast cancers.
Evaluation of metastases

Hematoxylin and eosin (H&E)-stained slides were reviewed by a pathologist using a standard microscope. The slides contained paraffin-embedded sections of lung tissue from the mice. Each slide was reviewed blinded and the number of metastases was counted. To ensure accuracy, the metastases on each slide were counted twice.

Treatment of mice bearing breast tumors with BKM120 or anti-PD-1

When the PyMT or 4T1 tumors became palpable (~2 weeks), mice were treated for 3 weeks with BKM120 (30 mg/kg, every day) or vehicle by oral gavage. Tumor size (microlipimer) and mouse weight were measured weekly. For anti-PD-1 experiments, PyMT cells were delivered to C57Bl/6 mice and when tumors grew to 10 mm in length (~28 days) mice were treated with controls (DMSO and/or IgG2b), BKM120 (30 mg/kg, daily), PD-1 antibody (100 μg, on days 1 and 4 each week) for two weeks (day 42). Tumor size was measured twice weekly with microcalipers and tumor volume was calculated as 0.5 × length × width × depth. After mice were euthanized, final tumor volume was determined by fluid displacement. Aliquots of tumor and lung were processed for FACS analysis of infiltrating leukocytes, for RNA or protein analysis, and for histology (see details in Supplementary Methods).

Statistical analysis

Experiments designed to evaluate treatment effects on tumor growth were analyzed using mixed-effect models (Figs. 1A and 4A, Supplementary Methods). The Wilcoxon rank-sum test or the growth were analyzed using mixed-effect models (Figs. 1A and 4A).

Results

BKM120 efficiently inhibited mammary tumor growth and metastasis

To evaluate the effects of a pan-class I PI3K inhibitor in a mammary tumor treatment model, BKM120 or vehicle were delivered to C57Bl/6 mice once palpable tumors were detected (about two weeks after orthotopic implantation of MMTV-PyMT mammary tumor cells (3 × 106). BKM120 (30 mg/kg), or vehicle alone, was delivered to mice (n = 5/group) daily by oral gavage for 3 weeks. Tumors grew rapidly in vehicle control mice, while tumors in the mice treated with BKM120 stopped growing or grew very slowly (Fig. 1A, P < 0.0001 mixed-effects model with a heterogeneous variance correction model for repeated measures with mean contrasts; day 26, P = 0.0001, day 33, 36, and 40, P < 0.0001). Western blot analysis of representative mouse tumors and lungs revealed that the BKM120 did inhibit the level of p-AKT (S473; Fig. 1A, bottom). Moreover, histologic measurements of metastases in the lungs (Fig. 1B, bottom) of tumor-bearing mice showed a reduction in the tumor weight and a reduction in the number of metastases in the BKM120-treated mice after 3 weeks of treatment compared with vehicle control (Fig. 1B (P = 0.0088, P = 0.0071, respectively, Wilcoxon rank-sum test). We did not detect (P < 0.05) an association between tumor weight and number of metastases, adjusted for treatment (P = 0.2948, multivariable linear regression).

Similarly, we performed experiments in BALB/c mice with orthotopic implants of 4T1 breast cancer cells (5 × 106 cells) to examine the effects of BKM120 (30 mg/kg daily, n = 10) versus vehicle control (n = 10). We observed no significant difference in tumor weight in the BKM120-treated versus vehicle control groups (P = 0.06, two-sample t test on a log2 scale), but there was a significant decrease in lung metastasis (P = 0.0035, two-sample t test on a log2 scale; Fig. 1C and D) in the BKM120-treated group. Moreover, there was no significant linear association between tumor weight and number of metastasis, adjusted for treatment (P = 0.6228 on a log2 scale). Also, there was no linear association between tumor weight and number of metastasis among BKM120-treated group (P = 0.5739 on a log2 scale) as well as among vehicle (P = 0.8661 on a log2 scale) treated group.

We performed neutrophil chemotaxis assays (CXCL8 ligand, 0, 30, 300 ng/ml) in the presence or absence of BKM120 (0–1,000 nmol/L) and as expected from our prior work, the migration of neutrophils isolated from mouse bone marrow was not significantly affected by PI3K inhibition (Fig. 1E; refs. 33, 34). To determine how BKM120 treatment affected tumor cell migration and invasion, we performed in vitro transwell invasion assays. Interestingly, the invasion of MMTV-PyMT mammary tumor cells toward 100 ng/ml of CXCL12 was dramatically inhibited by BKM120 treatment (Fig. 1F, P < 0.001 for 300 nmol/L of BKM120 vs. control and P < 0.001 for 1,000 nmol/L of BKM120 vs. control). These data suggest that tumor cell extravasation from the blood vessels into the lung could be compromised by BKM120 treatment to reduce metastasis.

Leukocyte population infiltrated in primary tumor and lung

The leukocyte infiltrate is a key feature of the tumor stroma. The cytokine profile and composition of leukocyte infiltrate may turn the microenvironment into a "protumor" or "antitumor" environment. Accordingly, we examined the changes in the intratumor leukocyte population when PI3K was systematically inhibited by BKM120. While there were no significant changes in the total percentage of immune cells (CD45+) in the tumor or lung after BKM120 treatment at the time tumors were harvested (Fig. 2A), when subpopulations of leukocytes in the tumor were examined, there were significant decreases in the number of macrophages (CD11b+/CD11c+/F4/80+) in the tumor, but not the lung (Fig. 2B). Moreover, BKM120 treatment resulted in an increase in the number of CD4+ cells in the primary tumors (P = 0.05), but not the lungs, with a trend toward an increase in CD8+ T cells in tumor, but not lung (Fig. 2B). BKM120 treatment also produced an increase in the percentage of IFNγ-expressing CD4+ T cells in the tumor (Fig. 2C, P = 0.05), indicating a shift toward a Th1 phenotype. These group comparisons were made using two-sample t test.

Tumor cells can be killed directly by cytotoxic lymphocytes. To evaluate the effect of BKM120 treatment on T-cell killing of tumor cells, T-cell proliferation and cytotoxicity toward tumor cells was tested in vitro (35, 36). Treatment of splenic cells with 300 nmol/L BKM120, a concentration equivalent to the in vivo dosage used in these experiments, resulted in significant inhibition of CD4+ (P < 0.001), but not CD8+ T-cell proliferation.
In other experiments, BALB/c mice were injected with 4T1 cells (1 × 10^6) then 3 days later BKM120 (30 mg/kg) treatment was initiated. After 7 days of treatment, CD8^+ T cells were isolated from the spleen and cytotoxicity assays were performed over an effector-to-target ratio of 0:1 to 50:1 (50 T cells for every 1 tumor cell). BKM120 did not significantly reduce the cytotoxicity of T cells toward target 4T1 tumor cells, \( P > 0.05 \), Wilcoxon rank-sum test.
Figure 2.
Type I T-cell populations are increased in both tumor and lung of BKM120-treated mice bearing PyMT tumor 5 weeks after tumor cell implantation. **A**, Compared with the vehicle control group, there was no change in the overall number of immune cells in the tumor or the lung after BKM120 treatment for 5 weeks. **B**, Number of different subsets of immune cells in tumor tissue (left) and lungs (right) in percent of immune cells (CD45) in mice treated with BKM120 versus vehicle based on FACS analysis. **C**, Number of CD4+ IFNγ+ cells based on intracellular analysis via FACS in BKM120-treated mice versus vehicle. Group comparisons were made using Wilcoxon rank-sum test.

Effects of BKM120 on the leukocyte population in the pre-metastatic niche

BKM120 inhibited 4T1 tumors in the formation of lung metastasis in BALB/c mice (Fig. 1C) with a significant week by treatment effect \( (P < 0.0001, \text{mixed-effects model with heterogeneous variance correction on a nature log scale}) \). To evaluate the possibility that BKM120 altered the premetastatic niche of the lung, nontumor–bearing BALB/c mice were treated with vehicle or BKM120 (30 mg/kg, daily) for two weeks, then the leukocyte population in the lungs was analyzed by FACS. Four different populations of cells in the lung were identified on the basis of expression of Ly6C and Ly6G on CD11b+ cells. There was a significant increase in the accumulation of CD11b+Ly6C+Ly6Glow leukocytes in the lungs of mice treated with BKM120 \( (P = 0.005, \text{two-sample } t \text{ test}) \) and there was a decreased number of Ly6GlowLy6C+ cells (Fig. 3A). No differences in the number of T cells in the lung in response to BKM120 treatment were observed (two-sample \( t \) test).

Analysis of the gene expression profile of representative cytokines and chemokines (Fig. 3B) from CD11b+Ly6C+Ly6Glow cells isolated from mouse lungs of nontumor–bearing mice revealed BKM120 increased IL6 and CXCL11 and decreased CCL5 (RANTES) mRNA expression in the Ly6C+ cells isolated from the mouse lung. These data suggest the Ly6C+ cells may trend increasingly toward an antitumor (antimetastatic) phenotype in the lung tissue after BKM120 treatment. Analysis of the phenotype of these cells showed changes in MHCII expression. Ly6C+ cells contain two subpopulations of cells, MHCI+ cells and MHCII+ cells, and BKM120 treatment decreased the number of MHCII+ cells as well as the expression of MHCII (Fig. 3C). No significant changes were found in surface expression of CX3CR1, CXCR7, CXCL11, CD54a (α1), β6, CD49e (α5), CD51, CD197, CD61, CD49f (α6), CD126, F4/80, β6, CD49b, CD124, CD80, CD49f, CD184, CD49d, CD86, CD244, CD182, CD81, CD11c, CD115, and CD104 (data not shown).

To determine whether BKM120 was affecting the differentiation of mouse or human bone marrow cells into Ly6C+ cells, in vitro experiments were performed. Bone marrow cells were differentiated into myeloid cells in the presence of GM-CSF (20 ng/mL) with either BKM120 or vehicle for 5 days. Results show that BKM120 treatment of mouse and human bone marrow cells resulted in an approximately 25% increase in Ly6C+ (mouse) and CD14+CD1a+ (human) cells, respectively (Fig. 3D). Altogether, these data indicate that BKM120 can influence the differentiation of bone marrow progenitors and can increase the Ly6C+ population of cells in the premetastatic niche of the lung, shifting these cells toward a more proinflammatory phenotype to impair metastasis formation (Fig. 3).

The inhibition of tumor growth and metastasis is diminished if BKM120 treatment is paused then reinitiated

One possible explanation for the reduction in tumor metastasis in mice treated with BKM120 is that the primary tumors were smaller in size and number in the treatment group than the tumors in the control group and as a result had not reached sufficient size for metastasis. To test this possibility, we allowed PyMT tumors in BKM120-treated C57Bl/6 mice to grow to the same size as the control group prior to sacrifice and analyzed
metastatic lung lesions. In another group of mice, BKM120 treatment was paused for two weeks and then either reinitiated or not reinitiated in the two treatment subgroups. For treatment-paused mice, tumors grew rapidly after suspension of BKM120 treatment, and once treatment resumed, rapid tumor growth continued. Tumors from mice that received continuous BKM120 treatment for 7 weeks exhibited a growth curve that was slower than the vehicle control group, when we compared the linear component of the two growth curves ($P = 0.005$; Supplementary Fig. S2). There was no difference in number of metastatic lesions in the lung for all the groups of mice that received different periods of BKM120 treatment (data not shown). However, one caveat for this experimental design is that the BKM120-treated tumors have been growing for a longer time, thus allowing increased time for metastasis, so we cannot conclude from this experiment that metastasis frequency is directly related to tumor size. Moreover, as noted in the 4T1/BALB/c model in Fig. 1C and D, even within the vehicle

Figure 3.
Increased accumulation of Ly6C$^+$ leukocytes in lungs in mice pretreated with BKM120. A, Significantly increased lung accumulation of CD11b$^+$ Ly6C$^+$ Ly6G$^{low}$ leukocytes in the mice pretreated with BKM120. Nontumor-bearing BALB/c mice (9-week-old females) were treated with vehicle or BKM120 (30 mg/kg, daily) for two weeks; mice were sacrificed and then single-cell suspensions were generated from lungs and FACS analysis was performed. Plots are gated for CD45$^+$CD11b$^+$ cells. No differences were found in T-cell number (plots are gated for CD4$^+$CD3$^+$ cells). B, Gene expression profile (quantitative real-time PCR) from lungs of mice treated with BKM120 as described above in A (*, $P < 0.05$). C, MHCII expression on CD11b$^+$Ly6C$^+$Ly6G$^{low}$ leukocytes in lungs. The in vitro differentiation of myeloid cells from mouse bone marrow (top) and human bone marrow (bottom) in presence of GM-CSF, 20 ng/mL and BKM120, 300 nmol/L.
Effects of combined BKM120 and anti-PD1 on the growth of PyMT tumors

BKM120 treatment of mice bearing breast tumors results in alteration of immune cells in the tumor microenvironment. We hypothesized that combining BKM120 treatment with the checkpoint inhibitor, anti-PD1, would result in enhanced reduction in tumor growth. First, however, we evaluated whether inhibition of PI3K would affect PD-L1 expression by tumor cells or PD-1 expression by T cells. We treated MMTV-PyMT tumor cells with IFNγ and either BKM120 or vehicle for 24 hours then examined the surface expression of PD-L1 in MMTV-PyMT tumor cells. We did not see any changes in PD-1 expression on MMTV-PyMT cells after BKM120 treatment (Supplementary Fig. S3A). We also observed no changes for the PD-1 expression in T lymphocytes after treatment with BKM120 or vehicle control and stimulated with the plant lectin Concanavalin A, a mitogen for mouse T-cell subsets, for 3 days in vitro (Supplementary Fig. S3B).

Based upon these results, we determined whether combining BKM120 and anti-PD1 would result in greater inhibition of tumor growth than with either agent alone. C57BI/6 female non-parous mice (10 weeks of age) were implanted orthotopically into the fourth mammary gland with ∼50,000 syngeneic PyMT cells. When tumors reached a size of approximately 1 cm in any direction (~4 weeks), mice were either treated with Vehicle, IgG, vehicle + IgG, or +PD-1-blocking antibody (100 μg twice a week on days 1 and 4). BKM120 (50 mg/kg daily) or a combination of anti-PD-1 and BKM120 for two weeks. Tumor measurements over the treatment period showed significant reduction in growth with BKM120 alone [Holm adj P < 0.0001, mixed-effects model comparing least square (model predicted) means, Fig. 4A] and the addition of anti-PD1 resulted in a significantly smaller mean tumor volume than with BKM120 treatment alone (Holm adj P < 0.001=0.0002, mixed-effects model as above, Fig. 4A; n = 10). Comparisons between average tumor volume (∆SE) at the 14th day revealed that differences in average tumor volume between BKM120 + anti-PD1 and BKM120 were statistically significantly (Holm adj P < 0.011), while differences for each in comparison with vehicle control or vehicle plus IgG2b were also statistically significant (Holm adj P < 0.001 and adj P < 0.001, one-way-ANOVA with model predicted mean contrasts, Fig. 4A right). The histology of the primary tumors typed PyMT tumors as advanced adenocarcinomas with enhanced presence of B cells, CD8 T cells, and FoxP3+ cells in BKM120 and the combined BKM120 and PD-1 antibody–treated tumors (Fig. 4B). Quantification of FoxP3 IHC for both peritumoral area and intratumoral area revealed distinctive accumulation of FoxP3+ cells in BKM120 (20× field of view) compared with combined treatment (Fig. 4C). FACS analysis of immune cells infiltrating the tumors after therapy revealed an increase in the leukocyte content in oPD-1, BKM120, or combined treatments. There were significant increases in B-cell, T-cell, and CD4/CD25+ cells in BKM120 alone treated tumors, and addition of anti-PD1 to the BKM120 treatment further increased the B-cell infiltrate and reduced the BKM120 increase in CD4/CD25+ cells (Fig. 4D; Supplementary Fig. S3C and S3D). There was no difference in the viability of immune cells between the four treatment groups. BKM120 alone and BKM120 + anti-PD1 tended to increase the NK-cell population (CD49b+ CD5+) as compared with control or anti-PD1 alone; the combination treatment also tended to reduce the neutrophil population (Ly6g/CD11b+) and increase the percentage of Ly6c+Ly6c+ CD11b cells. Each of the three treatments tended to increase F4/80/MHCII+ CD45 cells compared with vehicle control (Supplementary Fig. S3E). Also, immunohistochemical analysis of tumor tissues after treatment with anti-PD1, BKM120, or the combination revealed that the BKM120, but not anti-PD1 or the combined treatment increased the FoxP3 content around the edges of the tumor, while the combined treatment resulted in increased intratumoral FoxP3 cells. Neither of the treatments affected the splenic distribution of FoxP3 cells. Moreover, BKM120 increased the tumoral CD4+ T-cell content, but the combination treatment diminished this (Supplementary Fig. S3F).

BKM120 treatment results in increased desmoplasia and a fibrotic tumor microenvironment

One of the more notable histologic observations in tumors treated with BKM120 was the accumulation of reactive stroma (Supplementary Fig. S4A). BKM120-treated tumors also had a noticeable increase in collagen accumulation and maturation within the tumor as demonstrated by trichrome and picroSirius red staining (Supplementary Fig. S4A). An immunofluorescent staining of tumors revealed cancer-associated fibroblast (CAF) markers, αSMA and vimentin, were more densely distributed within the BKM120-treated tumors (Supplementary Fig. S4B). To investigate the effect of BKM120 on CAFs, we established three primary fibroblast cell lines from PyMT mouse tumors and treated these with BKM120 (1 μmol/L for 24 hours), or vehicle. Treatment of three cell lines established from 3 different mice resulted in a significant increase in Il4 and Ccl5 mRNA (Supplementary Fig. S4C, P < 0.05). Primary fibroblasts cultures established from mammary glands of PI3Kγ-null mice also had elevated Il4 and Ccl5 expression (Supplementary Fig. S4D, P < 0.05 and P < 0.01, respectively). When we established primary human fibroblast cultures from mammaplasty reductions we found that treatment with the PI3Kγ-specific inhibitor, AS605240, also significantly elevated Il4 and Ccl5 gene expression (Supplementary Fig. S4E, P < 0.5). Altogether these results indicate that reduced tumor growth resulting from PI3K inhibition is accompanied by a desmoplastic event that triggers an inflammatory tumor microenvironment.

Growth of orthotopic implants of human breast tumor tissue into humanized NSG mice is inhibited by BKM120

The findings with MMTV-PyMT mouse tumors prompted us to test how BKM120 affects tumor growth and antitumor immunity in a human breast cancer model. We used a "humanized mouse" model in which tumors from triple-negative breast cancer (TNBC) patients were orthotypically implanted into the mammary fat pad of NSG mice that were engrafted with the same patient’s CD34+ HSCs followed by infusion with the patient’s CD4 (hCD4) and CD8+ (hCD8) T cells (Supplementary Fig. S5 and Supplementary Methods). Tumor-bearing mice treated with BKM120 exhibited a reduction of tumor-infiltrating hCD4+ T cells (57%±4.4% vs. 30%±4.3%), but of the hCD4+ cells detected, there was an increase in the percentage that were activated (CD4+CD69+ cells, 3.7%±1.2% in vehicle vs. 38%±3.9% in BKM120 treated; Supplementary Fig. S6A). Similarly, the percentage of the human CD45+ cells that were human B-cells (hB) tended to increase in...
BKM120-treated engrafted mice (CD20+ cells, 3% ± 0.8% vs. 11% ± 3.4%), but the increase did not achieve statistical significance (Supplementary Fig. S6A). BKM120 did not affect the CD8+CD107+ T cells, which were quite scarce in the tumors. BKM120 inhibition of the PI3K signaling pathway promoted activation of hCD4+ T cells based upon CD69 staining (P < 0.01) and increased the infiltration of hB cells [Supplementary Fig. S6B], leading to a switch in the CD4+ T-cell lineage phenotype to antitumor immunity. There was a significant inhibition of tumor growth by the third and fourth weeks of treatment with BKM120 (P < 0.0001, mixed-effects model with heterogeneous variance correction [Supplementary Fig. S6C]). Similar

Figure 4. BKM120 is enhanced with combined anti-PD-1 immunotherapy. A, Eight-week-old C57Bl/6 female non-parous mice were implanted orthotopically into the #4 mammary gland with 5.0 x 10³ syngeneic PyMT cells. After 4 weeks when tumors reached 1-cm diameter, mice were treated with either a PD-1-blocking antibody (100 µg twice a week), BKM120 (30 mg/kg daily), or a combination of both anti-PD-1 and BKM120, for two weeks and then euthanized. There were 5 groups of control mice: 5 received DMSO (the solvent for the BKM120); 5 received a nonspecific IgG (control for the anti-PD1); and 5 received a combination of both (control for anti-PD1 and BKM120 treatments). Left, mean (SE) tumor volume over time. Measurements of tumor volume over time revealed significant reduction in growth with BKM120 treatment alone (adj. P < 0.001), while the tumors treated with the combination of BKM120 and anti-PD1 showed significantly greater reduction in growth and even regression [(adj. P < 0.001, n = 10)], P values from mixed-effects model, adjusted for multiplicity using the Benjamini and Hochberg method. Right, average tumor volume (SE) at the 14th day. Differences in average tumor volume between BKM120 + anti-PD1 and BKM120 were statistically significantly (adj. P < 0.01), while differences for each in comparison with vehicle control or vehicle plus IgG2b were also statistically significant (P < 0.001). P values were determined by ANOVA. B, Histology of primary tumors typifies PyMT tumors as advanced adenocarcinomas with enhanced presence of B-cells, CD8 T cells, and FoxP3+ cells in BKM120 and combined treated tumors. C, Quantification of FoxP3 IHC for both peritumoral area and intratumoral area revealed distinct accumulation of FoxP3+ cells in BKM120 (20× field of view) compared with combined treatment. D, FACS analysis of immune cells from primary tumors increased leukocytes in aPD-1, BKM-120 or combined treatments, with significant increases in B cells, T cells, and CD4+/CD25+ cells in BKM120 alone treated tumors, which was reversed with addition of aPD-1 combination. Error bars indicate SD and P values were determined by one-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001. Scale bars in histology indicate 100 µm.
results were obtained with a second TNBC engrafted and treated (data not shown). Therefore, the results with this humanized mouse model with an orthotopic PDX are similar to that with the PyMT and 4T1 mouse tumor models, where PI3K inhibition arrested tumor cell growth and provided a more antitumor milieu, although in the humanized mouse model, we saw a reduction in total CD4+ T cells and a shift to an antitumor phenotype (CD4+/CD69+) for the CD4+ T cells infiltrating the tumor.

The tumor growth and metastasis are inhibited in PI3K−/− mice
As PI3K is the major subtype involved in leukocyte chemotaxis, to investigate how loss of PI3K only in the nontumor cells of the host impacted tumor growth and metastasis, we evaluated the growth of the PI3K−/− wild type (WT) MMTV-PyMT tumor cells in PI3K−/− mice. We hypothesized that the loss of PI3K in the TME might alter recruitment of subsets of leukocytes to the tumor and lung, and therefore change the TME to affect growth of the primary tumor and metastasis to secondary organ sites. Five weeks after implantation of MMTV-PyMT into the mammary fat pad, the tumors in PI3K−/− mice grew much slower than those in the C57BL/6 PI3K+/WT wild-type mice (800 ± 75 mm3 in WT vs. 400 ± 50 mm3 in PI3K−/−, P < 0.01, two-sample t test; Fig. 5A). The number of lung metastasis was also significantly reduced in PI3K−/− mice, much like what was observed with BKM120 treatment (P < 0.01, two-sample t test; Fig. 5B). These results suggest that the loss of PI3K only in the host nontumor cells is sufficient to inhibit tumor growth and metastasis.

Figure 5. MMTV-PyMT murine mammary tumor growth and metastasis are inhibited in PI3K−/− mice. MMTV-PyMT mammary tumor cells (3 × 106) were implanted into the fourth mammary fat pad of PI3K−/− (n = 4) and WT C57BL/6 mice (n = 6) and tumor growth was monitored for 5 weeks after tumor cell implantation. A, Final tumor volume was determined by fluid displacement. B, Number of lung metastases were counted from H&E-stained sections of paraffin-embedded tissue of each perfused lung. C, Immune cells (CD45+) in tumor tissue and lungs by FACS analysis. D, Immune cell subsets in tumor tissue (left) and in lungs (right) by FACS analysis. All cell population calculated as percentage of immune (CD45+) cells. E, Number of IFNγ+ CD8 cells in lungs and (F) number of TNFα+ CD8 and CD4 cells in tumor tissue and lungs. Number of cells was calculated by intracellular staining of a single-cell suspension of tissue as described in Materials and Methods section. Statistics, two-sample t test.
PI3Kγ−/− mice exhibited increased leukocyte recruitment into the tumor compared with PI3Kγ WT mice

We observed similarly that with systemic PI3K inhibition by BKM120 treatment, the infiltration of PMN/IMC into the primary tumors was significantly increased in PI3Kγ−/− mice (Fig. 5C). Furthermore, significantly more CD4+ (P < 0.05), CD8+ T cells (P < 0.05), and CD19+ B cells (P < 0.05) were found in primary tumors in PI3Kγ−/− mice (Fig. 5D). In lungs of tumor bearing PI3Kγ−/− mice, the infiltration of lymphoid cells (CD8+ T cells or B cells) was significantly increased (CD8+ T cells P = 0.002; B cells P = 0.019) and there was also an increased number of monocytes (CD11b+ F4/80+ Gr1−, P = 0.019). Of note, as there are more lymphoid cells in the bone marrow and spleen of PI3Kγ−/− compared with WT mice (Supplementary Fig. S7A and S7B, Wilcoxon rank-sum test, P < 0.05), this could provide more lymphoid cells to infiltrate primary tumors and lungs. These data also suggest that PI3Kγ activity may play some role in suppressing the generation of lymphoid cells and when this is lost, these cells are increased in the spleen and bone marrow.

Also we found significant differences in IFNγ and TNFα secretion by CD8 and CD4 cells in tumor and lungs (Fig. 5E and F). Significantly more CD8 and CD4 cells were producing TNFα in tumor tissue and lungs from PI3Kγ−/− mice: (CD4+ T cells, P = 0.018 and CD8+ T cells, P = 0.001). A marginal effect was found in the production of IFNγ by CD8 cells in lungs of PI3Kγ−/− mice (Fig. 5E) and TNFα by CD8 cells in tumor tissue (Fig. 5E). To test whether PI3Kγ−/− loss in neutrophils and lymphocytes can impair the chemotaxis of these cells toward a gradient of chemokine, the chemotactic index of neutrophils isolated from bone marrow and of lymphocytes isolated from spleens of PI3Kγ−/− mice or WT mice were tested in a modified Boyden chamber assay (37). The chemotactic index in response to CXCL8 was not significantly different between the PI3Kγ−/− and PI3Kγ WT mice (Supplementary Fig. S7C), confirming our prior observations that leukocyte chemotaxis can occur in a PI3K-independent manner (33). Moreover, lymphocytes isolated from PI3Kγ−/− mice exhibited a migration toward CXCL12 that was not impaired and basal migration appeared to be enhanced compared with control (Supplementary Fig. S7D).

Depletion of CD8+ T cells resulted in partial restoration of tumor growth in PI3Kγ−/− mice

To determine whether the elevation in CD8+ T cells in PI3Kγ−/− mice contributed to the inhibition of tumor growth and metastasis to the lung, we depleted CD8+ T cells from these mice by intraperitoneal injection of anti-CD8 antibody (100 µg) for 3 consecutive days, then every other day for 3 weeks) and confirmed the depletion of CD8+ T cells by flow cytometry analysis of lung and spleen (Fig. 6A). In the lungs of WT mice, CD8+ cells as a percentage of total CD90+ cells were reduced from 29.5% ± 4.2% to 9.2% ± 2.6%, while in spleen, the reduction was from 12.9% ± 1.6% to 3.6% ± 0.9% after application of antibody for 3 weeks. In the lungs of PI3Kγ−/− mice, CD8+ cells decreased from 43.2% ± 1.4% to 4.6% ± 0.8%, while in spleen, the decrease of CD8+ T cells was from 16.5% ± 0.8% to 2.0% ± 0.3% (Fig. 6A for representative FACS analysis and graph showing data from each mouse). We also evaluated the effect of CD8 IgG on the CD4+ T-cell population. Differences in the CD4+ T-cell population in the lung (31.9 ± 1.7 (isotype control) versus 52.9 ± 1.1 (CD8 IgG) but not the spleen were observed in the PI3Kγ−/− mice after mice were treated with CD8 IgG, but there were no differences in the CD4+ cells in WT mice in the lung or the spleen (Fig. 6A)]. Interestingly, there was no statistical difference between growth rates of tumors in WT mice with or without CD8+ T-cell depletion. However, in PI3Kγ−/− mice, depletion of CD8+ T cells resulted in significantly larger tumor size than those without CD8+ T-cell depletion (P < 0.05 two-way ANOVA with Bonferroni post-tests at day 35). This finding indicates that the increased presence of activated CTL in a tumor microenvironment lacking PI3Kγ inhibits tumor growth. Although tumor growth in PI3Kγ−/− mice with depletion of CD8+ T cells resulted in enhanced tumor growth compared with isotype antibody control, the fact that growth equal to that of WT mice did not ensue suggests that other factors and other cells in addition to CD8+ T cells contributed to the inhibition of tumor growth (P < 0.001 two-sample t test P < 0.05 at day 24, P < 0.001 at day 31 and 35; Fig. 6B). As an additional issue, there was no statistically significant difference in lung metastasis frequencies of the mice treated with IgG control or anti-CD8 antibody which is likely related to the sparsity of CD8 T cells in control tumors (P > 0.05, two-sample t test; Fig. 6C). Notwithstanding these caveats, the crucial finding from these experiments is that loss of PI3Kγ in the tumor microenvironment alone results in an inhibition of tumor growth.

Discussion

PI3K activity is very important for both tumor progression and the immune cell activity, and systemic inhibitors of various PI3Ks have recently been developed for clinical use (17, 19, 38). Inhibition of PI3Kγ alone may not be effective in luminal breast cancer due to a rebound reactivation of class IA PI3K via PI3Kβ (39). In this study, we showed in three different orthotopic mammary cancer mouse allograft and xenograft models that treatment with a pan PI3K inhibitor, BKM120, markedly inhibited tumor growth and number of metastases while at the same time increased the migration of myeloid cells into the tumor. In the 4T1 and PyMT models, there was no statistical association between tumor growth and number of metastases, but in the PyMT model, if BKM120-treated tumors were allowed to grow to the same size as vehicle control prior to sacrifice, no difference in numbers of metastasis was observed. Thus, it is possible that in the PyMT tumors BKM120 appeared to decrease the number of metastases by decreasing primary tumor growth/burden. However, as these tumors were growing for a longer period of time, the loss of an effect on metastasis might be the result of a longer growing period. BKM120 did not significantly alter the number of CD8+ T cells migrating into the tumor, but did increase the percentage of intratumoral CD4+ T cells and of those a greater percentage produced IFNγ. Prior work from Marshall and colleagues support our findings that PI3Kγ can alleviate immune suppression by promoting IFNγ-secreting, antitumor T cells (40). We show that BKM120 also enhanced the differentiation of myeloid cells towards a monocytic lineage (Ly6C+Ly6G− F4/80− in mice, CD14+CD11b+ human) with an increased accumulation of these proinflammatory cells in the lungs of nontumor-bearing mice. At the same time, BKM120 impaired the invasive migration of tumor cells.
Our results also indicate that PI3K activity in the stromal cells of the tumor microenvironment plays a key role in tumor cell proliferation and migration. Loss of PI3K activity in the host was associated with dramatically increased infiltration of CD4⁺ and CD8⁺ T lymphocytes and PMN/iMC into the PI3K⁺/WT tumor; in lung, there was also a major increase in infiltrating CD8⁺ T lymphocytes, CD4⁺ T lymphocytes, and Tregs, but a decrease in PMN/iMCs and DCs that correlated with the decrease in lung metastasis. The tumor and lung-infiltrating CD8⁺ T cells expressed IFNγ and TNFα, indicating a strong antitumor environment was present. While we cannot rule out the possibility that some of the changes in the leukocyte populations are linked to decreased tumor size, as in other tumor models smaller tumors exhibit an influx of CD4⁺ and CD8⁺ T cells, which is followed by a decline in these T cells and an increase in neutrophils and macrophages as the tumor grows (41). Our results with PI3K⁺⁻ mice are somewhat similar to results from mice expressing inactive PI3Kδ (p110δ(D910A)) where growth and metastasis of B16 melanoma (C57Bl/6), Lewis lung carcinoma (C57Bl/6), and 4T1 breast cancer (BALB/c) were inhibited (10). Similar results were obtained with a P110δ-specific inhibitor. In addition, as we have shown with PI3K⁺⁻ mice, in their model depletion of CD8⁺ T cells removed the tumor response to the P110δ-specific inhibitor (10).

We report here that treatment with BKM120 efficiently inhibited MMTV-PyMT, 4T1, and TNBC PDX cancer growth and/or metastasis. Clinical trial results from Ma and colleagues reported that BKM120 was somewhat effective in treating ER⁺ breast cancer patients who did not exhibit loss of PTEN, loss of progesterone receptor expression, or mutation in TP53 (14). Moreover, response did not correlate with PI3KCA mutation and mutation in AKT1 or ESR1 did not ablate response (14). As is the case for other single-agent treatments for breast cancer, sustained PI3K inhibition results in development of tumor cell resistance to PI3K inhibitor therapy (15, 42).
To potentially avoid this resistance, we considered combining BKM120 with other agents, such as blockade of immune checkpoint inhibitors. In our studies with PI3Kγ-null mice, a depletion of CD8+ T cells partially restored the antitumor response against the PyMT tumors. Kaneda and colleagues showed that blockade of PI3Kγ reprograms tumor-associated macrophages toward a Th1 program to enhance CD8+ T-cell–mediated suppression of tumor growth and metastasis (41, 43). In fact, they showed that PI3Kγ activation results in a negative feedback to inhibit NF-kB–mediated production of key chemokines that recruit T cells (41).

Our findings also support the findings of Peng and colleagues that PI3K activity participates in development of resistance to T-cell immunotherapy (44). Considering our data in the context of work from Marshall and colleagues (40) and Peng and colleagues (44), it was be important to consider combining a pan-PI3K inhibitor with immune checkpoint inhibitors for treatment of breast cancers. Here we show that anti-PD1 was not effective as a monotherapy in the PyMT breast cancer model, but combining BKM120 with anti-PD1 further reduced tumor growth over that observed with BKM120 treatment alone and resulted in blockade of tumor growth in 100% of the mice. In contrast to our data, Kanada and colleagues showed that PI3Kγ inhibitor plus anti-PD1 was ineffective for treatment of pancreatic cancers in mice, but that combining PI3Kγ inhibitors with standard-of-care chemotherapy and anti-PD1 slowed the growth of pancreatic cancer (41). The difference between the two studies could be due to differences in use of a pan-PI3K inhibitor that will target breast tumor cells versus a PI3Kγ-specific inhibitor that does not directly target pancreatic tumor cells. Our data show for the first time that combining pan-PI3K inhibition with antibody to the checkpoint inhibitor PD1 markedly impairs breast tumor growth and induces tumor regression. These data should lead the way to new clinical trials combining anti-PD1 or anti-PD-L1 with pan-PI3K inhibitors for the treatment of TNBC, and perhaps allow lower doses of PI3K inhibitors to be used, as has been done with BKM120 and MEK combination therapy, thereby reducing toxicity (45).

Currently, we have limited information from clinical trials using checkpoint inhibitors in breast cancer. Pembrolizumab (anti-PD-1) has shown limited response in a phase Ib KEYNOTE-012 clinical trial in 32 TNBC patients with metastatic disease who had received up to 3 lines of prior chemotherapy and whose tumors stained positively for PD-L1 (1 complete response, 4 partial responses, and 7 stable disease). Three patients remained on pembrolizumab for 11 months, but there was one death related to treatment (46). Another trial with atezolizumab (anti-PD-L1) in 54 heavily treated TNBC patients showed an overall response rate of 24%, with 3 partial responses, and 7 stable disease). A number of clinical trials are ongoing to evaluate checkpoint inhibitors alone and in combination with chemotherapy in breast cancer, but the results are outstanding (48). IHC analysis of PD-L1 expression has been reported to be high for TNBC, 60% for membranous, 77% for cytoplasmic, and 93% for stromal staining (49). PD-L1 expression in luminal breast cancer is less frequent (44%; ref. 50). Transcriptomic meta-analysis of a dataset of over 5,000 breast cancers revealed PD-L1 expression was highest in basal, followed by HER2+ and lowest in luminal breast cancers (51). These data point to TNBC patients as likely to benefit from combined PI3K inhibitor therapy in combination with checkpoint inhibitors, although we cannot exclude a benefit for patients with hormone-positive disease with treatment of a PI3K inhibitor and anti-PD1.

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

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Correction: PI3K Inhibition Reduces Mammary Tumor Growth and Facilitates Antitumor Immunity and Anti-PD1 Responses

In the original version of this article (1), Fig. 4A has an error in the ordinate of the graph and is off by a factor of 10. The error has now been corrected in the recent online HTML and PDF versions of the article. The authors regret this error.

Reference

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PI3K Inhibition Reduces Mammary Tumor Growth and Facilitates Antitumor Immunity and Anti-PD1 Responses

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