Microenvironment and Immunology

Immunotherapy with PI3K Inhibitor and Toll-Like Receptor Agonist Induces IFN-γ⁺IL-17⁺ Polyfunctional T Cells That Mediate Rejection of Murine Tumors

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Abstract

The immunosuppressive microenvironment in tumors hampers the induction of antitumor immunity by vaccines or immunotherapies. Toll-like receptor (TLR) ligands have the potential to treat tumors, but they can exert a mixture of positive and negative effects on inflammation in the tumor microenvironment. In this study, we show that specific small molecule inhibitors of phosphoinositide 3-kinase (PI3K) relieve immunosuppression to heighten the proinflammatory effects of TLR ligands that support antitumor immunity. Multiple strategies to inhibit PI3K in dendritic cells (DC) each led to suppression of interleukin (IL)-10 and TGF-β but did affect IL-12 or IL-1β induction by the TLR5 ligand flagellin. In three different mouse models of cancer, combining flagellin with a class I PI3K inhibitor, either with or without a DC vaccine, delayed tumor growth and increased survival, with some animals exhibiting complete rejection and resistance to secondary challenge. Tumor growth suppression was associated with increased accumulation of polyfunctional T cells that secreted multiple effector cytokines, including IFN-γ, IL-17, and IL-2. Therapeutic protection was abolished in mice deficient in IL-17 or deprived of IFN-γ. Together, our results indicate that PI3K inhibition heighten the antitumor properties of TLR ligands, eliciting tumor regression directly but also indirectly by relieving suppressive signals that restrict potent antitumor T-cell responses. These findings suggest important uses for PI3K inhibitors in heightening responses to cancer immunotherapy and immunochemotherapy. Cancer Res; 11. ©2011 AACR.

Introduction

The induction of effective antitumor immune responses is hampered by several factors, including the immunosuppressive nature of the tumor environment and poor infiltration of immune cells into the tumor mass. Antitumor responses can be induced in vivo by adoptive transfer of in vitro activated autologous dendritic cells (DC) primed to present tumor rejection antigens. Because DCs play a critical role in directing adaptive immune responses, this approach has considerable potential for generating antitumor effector T cells and the first DC-based cancer vaccine has recently been licensed (1). Nevertheless, tumor vaccines still have limited success in mediating tumor regression (2, 3).

Activation of toll-like receptors (TLR) on DCs leads to maturation and induction of cytokines that regulate adaptive immunity against pathogens and tumors. The TLR7/8 agonist, imiquimod is used in the treatment of superficial basal cell carcinoma (4). However, other studies with TLR agonists have met with limited success in treating human cancers (5). This may reflect the dual nature of responses induced by TLR agonists, with the production of both pro- and anti-inflammatory cytokines (5). This dual activity coupled with the suppressive tumor environment, replete with anti-inflammatory cytokines and regulatory T (Treg) cells, has limited wider clinical application of TLR agonists as tumor therapeutics.

The phosphoinositide 3-kinase (PI3K) signaling pathway is a lipid and protein kinases that control many cellular functions, such as growth, proliferation, and apoptosis (10) and consist of PI3Kα, PI3Kβ, PI3Kδ (class-IA), and PI3Kγ isoforms (class IB). Expression of PI3Kα and β is ubiquitous, whereas PI3Kδ and γ are expressed mainly by leukocytes (11).

PI3K is also activated by TLR signaling in innate immune cells, in which it seems to regulate inflammatory cytokine production (12, 13). However, the precise role for PI3K subtypes in DC activation and cytokine production remain unclear (12–16). In this study, we addressed the hypothesis that...
inhibition of specific isoforms of PI3K will inhibit anti-inflammatory cytokines, allowing enhancement of inflammatory cytokines, such as interleukin (IL)-12, thereby promoting anti-tumor immunity (17). We show that synthetic inhibitors of different PI3K isoforms not only mediate tumor cell killing, but in combination with TLR agonists promote IFN-γ- and IL-17-secreting T cells, leading to eradication of tumors in vivo.

Materials and Methods

Animals and cell lines
C57BL/6 mice were obtained from Harlan. IL-17-deficient (iL-17−/−) mice (18) were provided by Y. Iwakura (University of Tokyo, Japan). Use and care of mice was approved by the Trinity College Dublin ethics committee and the Irish Department of Health. B16.F10 melanoma, CT26.WT colon carcinoma, and [LL/2 (LLC1)] Lewis lung carcinoma (LLC) cell lines were obtained from the American Type Culture Collection (ATCC Standards).

PI3K inhibitors
Synthetic inhibitors of PI3K subtype signaling were purchased and used at concentrations determined by reference to manufacturers’ instructions and titrations to determine minimum concentrations to negatively affect secretion of IL-10 and positively affect secretion of IL-12p70 from flagellin-stimulated DCs (PI3-kinase α Inhibitor 2 (Cayman Chemical) was used at 10 nmol/L (19); PI3Kβ signaling was inhibited by the addition of 100 nmol/L TGX-221 (Cayman Chemical; ref. 20); PI3Kβ signaling was inhibited by addition of 10 nmol/L IC87114 (Caltag Medsystems; ref. 21); AS252424 (Cayman Chemicals) was used to inhibit PI3Kγ signaling by addition to cultures at 50 nmol/L (22). For inhibition of class I PI3K signaling, the pan class I PI3K inhibitor ZSTK474 was used at 50 nmol/L for 30 minutes prior to stimulation with TLR agonists. After washing, 5 × 105 to 105 cells were injected subcutaneously into the tumor site of the tumor-bearing mice with 1 to 20 μg/mL of an immunodominant CD8 T-cell peptide sequence (mTRP-218-218) (Genscript; ref. 28). After 72 hours cell proliferation was measured by 3H-thymidine incorporation and cytokine concentrations in supernatants were measured by ELISA.

DC activation and transfer
Mouse bone marrow–derived DCs were generated as previously described (25). DCs were pretreated with PI3K inhibitors for 30 minutes prior to stimulation with flagellin (100 ng/mL) from S. Typhimurium; Invivogen; dose determined by reference to manufacturers’ instructions and titration for induction of significant IL-12p70 and IL-10 production by DCs), and 1 hour before the addition of keyhole limpet hemocyanin (KLH; 5 μg/mL) or heat-shocked irradiated B16 tumor cells (hs/irB16) (26) at a 1 to 1 ratio to DCs. After washing, 5 × 105 to 105 cells were injected subcutaneously; IL-10, IL-12p70, TGF-β, and IL-1β (R&D Systems) concentrations were quantified in supernatant by ELISA. Intracellular cytokine expression was determined as described (27) using flow cytometric analysis on cells stained with anti-CD11c-PE-Cy5 (eBioscience), anti-IL-10-PE (eBioscience), and anti-IL-12p70-biotin (Becton Dickinson) followed by streptavidin A780 (eBioscience). DCs were also stained for expression of maturation markers using specific antibodies [MHC class II-FITC (fluorescein isothiocyanate), CD40-FITC, CD86-A780 (all eBioscience), CD80-PE (BioLegend), CD205-Alexa 647 (AbD Serotec)]. Cells were analyzed using Summit software on a CyanADP flow cytometer (Dako).

Immunoblot analysis for phosphorylated PI3K
DCs were stimulated as described above; after 15 minutes cells lysates were prepared and resolved on 12% SDS–PAGE gels and blotted onto Immobilon membrane (Millipore). Total PI3K p85, phospho-PI3K p85/55, total PI3K p110, p110β, p110δ, and pan-Actin were detected using the appropriate antibodies (Cell Signaling Technology and Santa Cruz Biotechnology).

Tumor challenge model
Mice were challenged with B16 melanoma (2 × 105 B16.F10 cells in C57BL/6 mice), CT26 carcinoma (5 × 105 CT26.WT cells in BALB/c mice), or LLC (1 × 105 LL/2 cells in C57BL/6 mice). All 3 tumor cell lines form solid tumors in mice when challenged subcutaneously with tumor cells into the flank. Tumor-bearing mice were treated with subcutaneous injections of 5 × 102 to 9 × 102 DCs or with direct injections (subcutaneously at the site of the tumor) of flagellin (200 ng/mouse; dose determined by titration for significant in vivo cytokine production following subcutaneous immunization) and/or class I PI3K inhibitor (0.042 or 0.42 mg/mouse) on days 3, 10, and 17 posttumor induction. In certain experiments, mice were injected intraperitoneally every 2 days from day -1 with 0.5 mg of anti-mouse IFN-γ antibody (clone XMG1.2).

T-cell responses
Proliferative lymph node (1 × 105/mL) from mice 7 days after DC transfer into the footpad were restimulated with KLH (10 μg/mL) for 72 hours before IFN-γ, IL-10, and TGF-β concentrations were detected in supernatants by ELISA, or cells stained for intracellular cytokines. Tumor-specific T-cell responses were tested by stimulating splenocytes from tumor-bearing mice with 1 to 20 μg/mL of an immunodominant CD8 T-cell peptide sequence (mTRP-2180-1885 Genscript; ref. 28). After 72 hours cell proliferation was measured by 3H-thymidine incorporation and cytokine concentrations in supernatants were measured by ELISA.

The phenotype of tumor-infiltrating lymphocytes was determined by flow cytometric staining tumor cells on day 22 posttumor induction. Single-cell suspensions of tumors were stimulated with phorbol myristate acetate and ionomycin (Sigma) for 2 hours prior to the addition of brefeldin A (5 μg/mL) for 4 hours. Cells were then stained for expression of CD3, CD4, CD8, IFN-γ, IL-2, IL-17, and IL-10 using the Intrastain kit (Dako). Cytokine producing cells were acquired using a CantoII cytometer (BD). Analysis and presentation of distributions was done using FlowJo v9.2 (Tree Star Inc) and SPICE version 5.1, downloaded from National Institute of Allergy and Infectious Diseases Exon Website (29, 30).

Statistical analysis
Statistical analysis was done using SigmaPlot 11 software (Systat Software). Kaplan–Meier survival curves of mice in the tumor challenge model were analyzed by the log rank survival test. Differences between multiple groups were compared using a one-way ANOVA with the Holm–Sidak method for all pair wise comparisons (normally distributed data) or the Kruskal–Wallis one-way ANOVA on ranks with the Tukey test method for multiple pair wise comparisons (nonnormally distributed data).
Results

PI3K controls TLR-induced regulatory cytokine production by DCs

We examined the involvement of PI3K signaling in the activation of DCs with a range of TLR agonists. Stimulation of murine bone marrow–derived DCs with the TLR2 agonist, zymosan, the TLR3 agonist, Poly I:C, the TLR4 agonist, lipopolysaccharide (LPS), the TLR5 agonist, flagellin or the TLR9 agonist, CpG induced secretion of varying concentrations of IL-12p70 and IL-10 (Fig. 1A). Addition of a PI3K class I inhibitor significantly suppressed secretion of IL-10 by DCs stimulated with TLR2, TLR4, and TLR5 agonists, and significantly enhanced IL-12p70 in response to TLR2 and TLR5 activation. In contrast, inhibition of PI3K enhanced IL-12p70 and IL-10 production by TLR9-stimulated DCs and suppressed TLR3-induced IL-12p70 secretion (Fig. 1A). Because flagellin induced IL-12p70 and IL-10 production, the latter suppressed by the PI3K inhibitor, and because it has been shown to be a safe and effective infectious disease vaccine adjuvant in humans (31), this TLR5 agonist was chosen for further investigation, with a view to using it as an adjuvant for a cancer vaccine.

Figure 1. Inhibition of PI3K suppresses TLR5-induced IL-10 from DCs. A, murine bone marrow–derived DCs were stimulated with TLR2 ligand, zymosan (Z; 25 μg/mL), the TLR3 ligand, Poly I:C (I; 200 μg/mL), the TLR4 ligand, LPS (L; 100 ng/mL), the TLR5 ligand, flagellin (F; 100 ng/mL) or the TLR9 ligand, CpG (C; 25 μg/mL) in the presence and absence of a class I PI3K inhibitor (0.025 μmol/L). After 24 hours of culture, IL-10 and IL-12p70 concentrations were quantified in supernatant by ELISA. **P < 0.01, and ***P < 0.001 for TLR ligand and PI3K inhibitor versus TLR ligand alone (t test). B, IL-12p70 and IL-10 concentrations quantified by ELISA in supernatants of DCs stimulated with flagellin (FLG) in the presence and absence of specific inhibitors of PI3K α, β, δ, and γ. ***, P < 0.001 versus untreated DCs. C, intracellular cytokine staining (IL-12p70 and IL-10) of CD11c+ cells after 24 hours stimulation with flagellin ± indicated PI3K inhibitors. Results are mean (±SD) percentage of CD11c+ cells expressing IL-12p70 and IL-10 from 4 experiments. ***, P < 0.001, and ****P < 0.001 versus flagellin alone. D, expression of PI3K p110, p110β, p110δ, p85, phosphorylated (Phos) p85, and β-actin in DCs stimulated with medium (−), flagellin alone or flagellin in the presence of PI3K inhibitors (α, β, δ, and γ, and class-I), determined by Western blotting.
We first confirmed that DCs expressed TLR5 using flow cytometry analysis (Supplementary Fig. S1A). We next examined the effect of a range of PI3K inhibitors with specificity for different isoforms of PI3K on TLR5-induced cytokines by DCs. Like the PI3K class I inhibitor, inhibitors of PI3Kα, β, δ, and γ, significantly suppressed IL-10 secretion by flagellin-stimulated DCs (Fig. 1B and C). Inhibition of PI3Kβ or class I signaling also suppressed IL-10 production, whereas inhibition of PI3Kβ or δ or class I did not significantly alter IL-12 production detected by ELISA (Fig. 1B). These findings were confirmed over a range of doses of inhibitors; each of the inhibitors suppressed IL-10 production by flagellin-activated DCs and PI3Kα and γ, but not β or δ or class I suppressed IL-12 production over a wide dose range (Supplementary Fig. S2). Intracellular cytokine staining confirmed that inhibitors of PI3Kα, β, δ, and γ significantly suppressed IL-10 production, whereas inhibition of PI3Kβ or δ significantly enhanced IL-12, and inhibition of PI3Kα or γ significantly suppressed IL-12 production (Fig. 1C). DC maturation was also largely unaffected by inhibitors of PI3Kβ or δ (Supplementary Fig. S3).

Class IA PI3K are composed of a p85 or p55 regulatory and a p110 (p110α, β, or δ) catalytic subunits. Flagellin activation of DCs promoted phosphorylation of PI3K p85, as shown by Western blot analysis with an antibody specific for phosphorylated PI3K p85 and p55 regulatory subunits (phosphorylation of p85 was not detected; Fig. 1D). Inhibitors of PI3Kβ, δ, γ, and class I, but not PI3Kα, attenuated flagellin-induced phosphorylation of PI3K p85 (Fig. 1D, Supplementary Fig. S4). Expression of total p110 or p110β or δ was not appreciably affected by any of the inhibitors examined. Although expression of total p85 was reduced by PI3Kβ and class I inhibitors, expression of phospho p85 relative to total p85 was reduced by inhibitors of PI3Kβ, δ, γ, and class I (Supplementary Fig. S4). These results suggest that TLR agonist–induced signaling via PI3Kβ and δ seems to be critical for the induction of IL-10 and TGF-β, but not IL-12 or maturation signals in DCs.

Inhibition of PI3K in DCs modulates their ability to induced T-cell response in vivo

Because activation of DCs with flagellin in the presence of a PI3K inhibitor induced a cytokine profile of low IL-10 and TGF-β and high IL-12, these DCs should promote the induction of Th1, but not Treg cells. We tested this hypothesis using a DC-adoptive transfer model, in which DCs were activated in vitro with flagellin and the model antigen, KLH, in the presence or absence of a class I PI3K inhibitor and injected subcutaneously into naive mice. Assessment of KLH-specific T-cell responses in the lymph node 7 days later revealed that transfer of KLH-pulsed and flagellin-stimulated DCs to naive mice induced KLH-specific T cells that secreted IFN-γ, IL-10, and TGF-β (Supplementary Fig. S5). In contrast, the responses induced by transfer of DCs treated in vitro with KLH, flagellin, and PI3K inhibitor was more Th1-polarized, with high levels of KLH-induced IFN-γ and significantly lower antigen-induced IL-10 or TGF-β. Thus inhibition of PI3K suppresses innate IL-10 and TGF-β production by TLR5-activated DCs, and allowed them to selectively prime Th1 cells in vivo.

Inhibition of PI3Kβ and δ signaling modulates cytokine production by DC tumor vaccine

We next examined the ability of PI3K inhibitors to modulate cytokine production by TLR-activated DCs pulsed with killed tumor cells. DCs pulsed with flagellin and hs/irB16 produced significant levels of the proinflammatory cytokines IL-12p70 and IL-1β, but also produced high levels of anti-inflammatory molecules, IL-10 and TGF-β (Fig. 2A and B). Treatment of DCs with PI3K inhibitors (β or δ or pan class I) significantly reduced production of IL-10 and TGF-β in response to flagellin and hs/irB16, although having no effect on production of IL-12p70, IL-1β (Fig. 2A and B) or IL-23 (data not shown). Although the PI3Kδ and class I inhibitors did enhance expression of CD86, and the PI3Kβ inhibitor reduced expression of MHC class II, in general inhibition of PI3K signaling did not adversely affect expression of maturation markers by flagellin-activated DCs (Fig. 2C).

Inhibition of PI3K signaling in DCs significantly enhances their antitumor efficacy

We next examined if inhibitors of PI3K could improve the efficacy of DC-based tumor immunotherapy using an experimental model of melanoma. Treatment of tumor-bearing mice with DCs pulsed in vitro with hs/irB16 alone or with flagellin showed minor but insignificant decreases in tumor growth when compared with control PBS-treated mice (Fig. 3A) or DCs treated with medium only (data not shown). In contrast, treatment with DCs pulsed in vitro with hs/irB16 and flagellin in the presence of PI3K inhibitor showed a significant decrease in tumor growth and significant increase in survival, when compared with all other groups (Fig. 3A and B). Indeed, 40% of mice completely rejected the tumor challenge following administration of DC vaccine with the PI3K inhibitor and were resistant to rechallenge with tumor (Supplementary Fig. S6), suggesting induction of potent and functionally active effector memory T-cell responses in these animals.

Mice treated with DC vaccine pulsed with B16 antigen, flagellin, and the PI3K inhibitor had significantly decreased numbers of tumor-infiltrating IL-10–secreting CD4+ and CD8+ T cells and increased IFN-γ–secreting T cells (Fig. 3C and D). These results showed that protection induced with DC vaccine pulsed with hs/irB16, flagellin, and class I PI3K inhibitor was associated with augmented antitumor effector T-cell responses in vivo.

Direct administration of PI3K inhibitor and TLR agonist induces antitumor immunity

We next tested the possibility that both the immunomodulatory and tumor cell killing effects of PI3K inhibitors could be exploited in vivo by combining direct injections of a PI3K inhibitor with a TLR agonist using 3 distinct tumor models. We first showed that inhibition of class I PI3K signaling resulted in rapid cell death of B16 melanoma cells, CT26 carcinoma cells, and LLC cells in vitro (Fig. 4A). We next
assessed the ability of PI3K inhibitor to modulate TLR-induced innate cytokine production in vivo, using a sepsis-type model, based on LPS-induced serum cytokines. Injection of the TLR4 agonist LPS induced detectable concentration of IFN-γ, IL-12p70, IL-10, and TNF-α in the serum 4 hours later. Coinjection of the PI3K inhibitor significantly decreased LPS-induced IL-10, did not affect IL-12p70, and significantly enhanced TNF-α and IFN-γ concentrations in the serum (Supplementary Fig. S7). This is consistent with the in vitro data using DCs (Figs. 1 and 2) and shows that the PI3K inhibitor has immunomodulatory effects on TLR-induced responses in vivo.

We next examined effect of coadministration of flagellin and PI3K inhibitor on tumor growth in vivo using 3 distinct models (B16 melanoma, CT26 carcinoma, and LLC). Treatment, by subcutaneous administration at the tumor site, with PI3K inhibitor or flagellin alone induced a modest delay in tumor growth in all 3 models, but only in the LLC model did single treatments with flagellin or PI3K inhibitor significantly affect survival of mice. In contrast, treatment of tumor-bearing mice with a combination of the PI3K inhibitor and flagellin significantly decreased tumor growth and significantly increased survival in all 3 models (Fig. 4B and C). All mice treated with the PI3K inhibitor and flagellin rejected the LLC. In the B16 model, mice that completely rejected the tumors were found to be resistant to rechallenge with the tumor (Supplementary Fig. S8). These findings suggested that the combination of PI3K inhibitor and flagellin is a highly effective therapy for inducing antitumor immunity.

**PI3K inhibitor promotes tumor-specific T-cell responses by acting directly on the tumor cell and by modulating TLR-induced cytokine production by DCs**

To confirm our hypothesis that direct cotreatment with PI3K inhibitor and flagellin was acting to both directly kill tumor cells and to modulate antitumor immune responses, we examined the requirement for class I PI3K inhibitor and flagellin at both the tumor killing stage and the immune initiation stage of treatment. B16 melanoma cells were treated in vitro with either flagellin and/or class I PI3K inhibitor for 24 hours before being used as a source of antigen for DCs that were also treated with flagellin and/or PI3K inhibitor. In addition, heat shock and irradiated B16 cells were also included as a source of antigen to serve as a positive control. These DCs were then used to stimulate spleen cells (as a source of memory T cells) taken from mice 40 days after injection of tumors, which had been rejected by treatment in vivo with subcutaneous injections of PI3K inhibitor and flagellin. When either unstimulated or FLG-activated DCs were used to present any of the tumor cell preparations (hs/ir, flagellin, PI3K inhibitor, or flagellin and PI3K inhibitor treated-B16 cells), the dominant response was the secretion of IL-10 (Fig. 5). In contrast, DCs that were activated in vitro with PI3K inhibitor...
alone or in combination with flagellin were able to induce significant IFN-γ and proliferative responses, with low IL-10 production (Fig. 5). This Th1 response was dependent on the tumor cells serving as a source of antigen having been efficiently killed by either heat shocking and irradiating or being exposed to PI3K inhibitor, as B16 cells treated with flagellin alone did not induce such responses (Fig. 5). Indeed, tumor cells killed by PI3K inhibitor treatment were capable of

Figure 3. Inhibition of PI3K signaling enhances the efficacy of TLR-activated DC vaccines against tumors in vivo. B16 tumor-bearing mice were treated on days 3, 10, and 17 postinduction with DCs treated in vitro with the indicated combinations of hs/rB16, flagellin, and/or class-I PI3K inhibitor. A, mean (±SEM) tumor growth rates for 20 mice per group, combined from 4 experiments. ***P < 0.001 versus all other groups. ANOVA. B, Survival plots of tumor-bearing mice treated as in A. ***P < 0.001 versus all other groups. C, examples of intracellular IL-10 and IFN-γ production by tumor-infiltrating CD4+ and CD8+ T cells. D, frequency of intracellular IFN-γ and IL-10–secreting tumor-infiltrating T cells for mice in each treatment group, horizontal lines represent mean responses for each group. ***P < 0.001 versus all other groups; *P < 0.05 versus PBS-treated and DC–hs/r-l-treated groups (Kruskal–Wallis).

Figure 4. Direct administration of PI3K inhibitor and TLR agonist induces antitumor immunity in 3 murine models. A, in vitro growth of B16, CT26, and LLC cells after treatment with medium or PI3K class I inhibitor. Tumor growth (B) and survival (C) of mice following injection of B16, CT26, or LLC cells and treatment on days 3, 10, and 17 posttumor induction with PBS, flagellin, class I PI3K inhibitor, or a combination of flagellin and class-I PI3K inhibitor, injected subcutaneously into the tumor site. Results are mean (SEM) values pooled from up to 4 experiments with 39 mice per group for B16, 16 mice per group for CT26, and 6 mice per group for LLC models. ***P < 0.001; **P < 0.01 versus PBS-treated group.
Recent reports have suggested polyfunctional effector T cells that simultaneously secrete multiple effector cytokines are more effective in killing tumor cells (32, 33) than cells that secrete IFN-γ alone. We therefore examined the possibility that treatment with PI3K inhibitor and flagellin could induce polyfunctional CD4\(^+\) and CD8\(^+\) T cells that secrete combinations of IFN-γ, IL-2, and IL-17 \textit{in vivo}. Tumor-infiltrating lymphocytes were stained with antibodies specific for surface CD3, CD8, and CD4 and intracellular IL-10, IFN-γ, IL-2, and IL-17 and fluorescence-activated cell sorting (FACS) analysis done and results analyzed using SPICE software. Control tumor-bearing mice showed very little polyfunctional T-cell activation, with tumor-infiltrating CD4\(^+\) T cells secreting IL-10 or IL-17, but little IFN-γ or IL-2 (Fig. 6B, Supplementary Figs. S9 and S10). In contrast, tumor-infiltrating CD4\(^+\) T cells from mice treated with flagellin and PI3K inhibitor produced significantly less IL-10 than tumor-bearing control mice and had increased numbers of polyfunctional tumor-infiltrating CD4\(^+\) T cells that simultaneously secreted combinations of IFN-γ, IL-2, and IL-17. In particular, we found a high frequency of IFN-γ-IL-17\(^{+}\), IFN-γ-IL-2\(^{+}\)IL-17\(^{+}\), IFN-γ-IL2\(^{+}\)CD4\(^+\) T cells infiltrating into tumors in treated mice. A similar pattern of responses was observed with tumor-infiltrating CD8\(^+\) T cells (Supplementary Fig. S11).

**IL-17 and IFN-γ are required for effective antitumor immunity**

Having shown that treatment with PI3K inhibitor and flagellin enhanced the infiltration of CD4 and CD8 T cells that secreted IFN-γ and IL-17, we examined the role of these cytokines in mediating the reduction in tumor growth. B16 tumors were induced in wild-type (WT) and il-17\(^{−/−}\) mice or mice treated with an anti-IFN-γ neutralizing antibody, and mice were treated with PI3K inhibitor and flagellin or PBS as a control. Treatment with PI3K inhibitor and flagellin significantly decreased tumor growth in WT mice but not in il-17\(^{−/−}\) mice or following neutralization of IFN-γ (Fig. 7A), showing that both IFN-γ and IL-17 are both required to mediate the antitumor effect of the combination immunotherapy (Fig. 7A).

Assessment of CD4\(^+\) T cells infiltrating into the tumor mass revealed that treatment with PI3K inhibitor and flagellin dramatically increased in the numbers of CD4\(^+\) T cells coproducing IFN-γ and IL-17 with no IL-10 (Fig. 7B and Supplementary Fig. S12). The frequency of IFN-γ-secerting tumor-infiltrating lymphocytes was significantly lower in flagellin and PI3K inhibitor treated IFN-γ-depleted compared with WT mice. However these mice still had a high frequency of IL-17-secreting T cells (Fig. 7B and Supplementary Fig. S12) and had a lower frequency of cells secreting IL-10 and IL-17 or IL-10 alone. Tumor-infiltrating CD4\(^+\) T cells from IL-17\(^{−/−}\) mice with flagellin and PI3K inhibitor lacked the high proportion of IL-17 and IFN-γ coproducing cells seen in WT flagellin and PI3K inhibitor-treated mice. However, CD4\(^+\) tumor-infiltrating lymphocytes from treated IL-17\(^{−/−}\) mice contained significantly more cells secreting IFN-γ alone than treated WT mice (Fig. 7B; Supplementary Fig. 12). Collectively, the data showed that neither IFN-γ-secreting T cells
in IL-17−/− mice nor IL-17–secreting T cells in IFN-γ–depleted mice were able to significantly reduce tumor growth, indicating a key role for both IFN-γ and IL-17 in PI3K inhibitor and flagellin treatment and providing further evidence that coproduction of these 2 effector cytokines is required for successful tumor rejection.

Discussion

The significant new finding of this study is that a TLR agonist and PI3K inhibitor, which have separately shown promise in treating tumors in humans, when combined are highly effective, either as a direct therapy or as part of a DC vaccine against a variety of murine tumors, through the combined effect of tumor killing and specific enhancement of polyfunctional T responses that secrete IFN-γ and IL-17.

The recent approval of Provenge by the U.S. Food and Drug Administration, for the treatment of asymptomatic or minimally symptomatic, hormone-resistant metastatic prostate cancer, suggests that the promise of active immunotherapies for cancer may finally be fulfilling its potential (1, 34). However, despite the approval of this first DC vaccine, and the large numbers of similar cell-based therapies and vaccines currently in advanced clinical trials (34), the objective outcomes of these treatment approaches fall well short of the desired cure for cancer. Despite the ability to induce antitumor immune cells in vivo, current immunotherapeutic approaches rarely induce tumor regression in advanced patients. Overcoming the immunosuppressive nature of the tumor is a particular challenge because we have to overcome local immune tolerance which allows immune system to prevent responses against self-antigens.

The approach described in this article, which exploits but modulates the ability of pathogen-derived molecules to induce potent immune responses, provides a new strategy to overcome tumor immunosuppression and poor immunogenicity of tumor antigens. The activation of innate immunity by TLR agonists and other PAMPs, although effective at driving adaptive immunity via IL-12 and IL-1, is counteracted by simultaneous induction of immunosuppressive cytokines, including IL-10 and TGF-β. We have identified a critical role for the PI3K signaling pathway in the production of the immunosuppressive molecules induced by TLR agonist activation of DCs. Indeed, we found that blocking PI3K signaling, especially PI3Kβ and δ, in DCs inhibited IL-10 and TGF-β production and modulated T-cell responses away from regulation and toward polyfunctional effector T cells that were able to mediate tumor killing and rejection in vivo.

Our findings in murine models are complemented by a recent study with human DCs, which has shown that blocking mTOR, a pivotal downstream mediator of the PI3K/Akt signaling pathway, promoted IL-12 and suppressed IL-10.
production in response to TLR-dependent and TLR-independent stimuli (35). This suggests that PI3K inhibitors can modulate TLR-induced cytokine production by human DCs, and combined with our study, suggests that they may also enhance antitumor immunity in humans. When coupled with the immune stimulatory effects of TLR agonists, the overall effect of inhibiting class I PI3K signaling directly in vivo was to promote effector T cells responses and tumor regression. Therefore, the use of PI3K inhibitors as a direct combination therapy with TLR agonist induced similarly effective tumor regression to the adoptive transfer of DCs primed in vitro with PI3K inhibitor and flagellin. However, direct administration has the advantage over DC transfer of not requiring expensive and time-consuming preparation of cells from individual patients and exploits the capacity of the PI3K inhibitors to mediate tumor cell apoptosis and cell-cycle arrest (9), allowing us to exploit their immunomodulatory and tumor cytotoxic effects.

It is well established that IFN-γ, produced by CD4, CD8, and innate immune cells, plays a major role in antitumor immunity (17). The role of IL-17–secreting T (Th17) cells and T cells coproducing IL-17 and IFN-γ is less clear. There is some evidence to suggest that IL-17–mediated inflammation may promote the development of tumors (36). However, it has also been shown that Th17 cells may function in tumor rejection either by acting directly on tumor cells or by activating and recruiting other immune cells that mediate tumor cell killing (36–38). We found that the PI3K inhibitor suppressed IL-10 and TGF-β, but not IL-1β, IL-12p70, or IL-23 by DCs, and this was consistent with the induction of IFN-γ and/or IL-17 producing T cells in vivo. Whereas TGF-β in combination with IL-6 induces differentiation of Th17 cells, IL-1β with IL-23 promotes IL-17 production by Th17 cells (39). Indeed, it is likely that inhibition of IL-10 and TGF-β are the critical factors attenuated by the PI3K inhibitor which enhance the potency of flagellin as an adjuvant. It has been shown that TGF-β and IL-10 promote induction of Treg cells (40, 41). In addition, we have shown that IL-10–defective DCs pulsed with TLR agonists and bs/ir tumor cells are more protective than similarly pulsed wild-type DCs (26). We have also reported that blocking TGF-β enhances Th1 and Th17 responses to infectious disease and autoantigens (42, 43). Our demonstration that IL-10+ Treg cells are reduced in vivo in mice treated with flagellin and the PI3K

Figure 7. Essential roles for IFN-γ and IL-17 in the antitumor responses induced by flagellin and PI3K inhibitor. B16 tumor-bearing C57BL/6 WT and IL-17−/− mice were treated with combinations of flagellin and PI3K inhibitor on days 3, 10, and 17 posttumor induction as described in Fig. 4. Additional groups of untreated and treated WT mice were injected intraperitoneally with anti-mouse IFN-γ neutralizing antibody every 2 days from day -1. A, mean tumor growth of B16 tumor-bearing mice. B, polyfunctional T cells analysis. Intracellular cytokine staining and FACS analysis on tumor-infiltrating lymphocytes (gated on CD3+CD4+ cells) with antibodies specific for, IL-10, IFN-γ, and IL-17. Polyfunctional T-cell populations are plotted according to the colors indicated in the Pie slice legend; T cells secreting combinations of cytokines detected at low frequency have been grouped and shaded grey and labeled "minor populations."
inhibitor, together with the finding that PI3K inhibitor suppresses IL-10 and TGF-β production by TLR5-activated DCs, is consistent with this conclusion.

We found that the antitumor effects of cotreatment with TLR agonist and PI3K inhibitor were dependent on both IFN-γ and IL-17. Treatment with flagellin and PI3K inhibitor substantially enhanced the frequency of IFN-γ+IL-17+ and IFN-γ+IL-17−IL-2− CD4+ T cells tumor infiltrating the tumors and this was associated with rejection or reduction in growth of the tumor. We do not have direct evidence that cells that coproduce IL-17 and IFN-γ mediate tumor rejection; however, we have shown nonredundant roles for IL-17 and IFN-γ. The protective effect of the combination therapy was abrogated in IL-17−/− mice or in mice treated with a neutralizing anti-IFN-γ antibody. The importance of polyfunctional T cells secreting multiple IL-17 effector cytokines in mediating antitumor effects is increasingly being recognized (32, 33). We found that production of IL-17 or IFN-γ alone by tumor-infiltrating T cells was not sufficient to mediate tumor rejection; significant numbers of IL-17− secreting T cells were detected in mice depleted of IFN-γ, and significant numbers of T cells secreting IFN-γ were detected in IL-17−/− mice, yet neither group of mice were capable of controlling the B16 tumors. These observations indicate that the antitumor effects induced with our combination therapy is mediated by T cells that secrete multiple effector cytokines, including IFN-γ and IL-17. Although IL-17 and IFN-γ coproducing cells have previously been identified, this is the first study to show a host protective role for these cells in the control of disease and suggests that they may be an important cell type to induce in the development of immunotherapeutic and vaccine approaches against cancer in humans.

Our finding show that PI3K inhibitors have considerable efficacy in modulating antitumor immune responses induced by TLR agonists in 3 distinct tumor models. This combination is an attractive approach for translation to the clinic, given its ability to mediate tumor regression via the induction of significant tumor-specific polyfunctional T-cell responses, even against a poorly immunogenic and aggressive melanoma tumor model.

Disclosure of Potential Conflicts of Interest

Kingston H.G. Mills is a cofounder, consultant, and shareholder in Opsona Therapeutics Ltd and TriMod Therapeutics Ltd, start-up companies involved in the development of immunotherapeutics.

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