Immunotherapy with PI3K inhibitor and Toll-like receptor agonist

induces IFN-γ⁺IL-17⁺ polyfunctional T-cells

that mediate rejection of murine tumors

Running title: PI3K inhibitors improve efficacy of TLR-based tumor vaccine

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Abstract

The immunosuppressive microenvironment in tumors hampers the induction of anti-tumor immunity by vaccines or immunotherapies. 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 PI3K relieve immunosuppression to heighten the pro-inflammatory effects of TLR ligands that support anti-tumor immunity. Multiple strategies to inhibit PI3K in dendritic cells (DC) each led to suppression of 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 anti-tumor properties of TLR ligands, eliciting tumor regression directly but also indirectly by relieving suppressive signals that restrict potent anti-tumor T cell responses. These findings suggest important uses for PI3K inhibitors in heightening responses to cancer immunotherapy and immunochemotherapy.
**Introduction**

The induction of effective anti-tumor 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. Anti-tumor responses can be induced *in vivo* by adoptive transfer of *in vitro* activated autologous dendritic cells (DC) primed to present tumor rejection antigens. Since DC play a critical role in directing adaptive immune responses, this approach has considerable potential for generating anti-tumor 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 DC 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, this has limited wider clinical application of TLR agonists as tumor therapeutics.

The phosphoinositide 3-kinase (PI3K) signalling pathway is an important target for tumor therapy by virtue of its role in cell survival and its dysregulation in tumors (6-7). Several inhibitors of PI3K or mTOR have already been tested in phase I and II clinical trials (7-9). The class-1 PI3K are a family of dual-specific 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 PI3K α and β is ubiquitous, whereas PI3K δ and γ are expressed mainly by leukocytes (11).
PI3K is also activated by TLR signaling in innate immune cells, where it appears 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 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*−/− 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 cell lines were obtained from the ATCC (LGC Standards).

PI3K inhibitors

Synthetic inhibitors of PI3K subtype signalling were purchased and used at concentrations determined by reference to manufactures’ instructions and titrations to determine minimum concentrations to negatively affect secretion of IL-10 and positively affect secretion of IL-12p70 from flagellin stimulated DC (PI3-kinase α Inhibitor 2 (Cayman Chemical, Michigan, US) was used at 10 nM (19); PI3K β signalling was inhibited by the addition of 100 nM TGX-221 (Cayman Chemical, Michigan, US) (20); PI3Kδ signalling was inhibited by addition of 10 µM IC87114 (Caltag Medsystems, UK) (21); AS252424 (Cayman Chemicals, Michigan, US) was used to inhibit PI3Kγ signalling by addition to cultures at 50 nM (22). For inhibition of Class I PI3K signalling, the pan Class I PI3K inhibitor ZSTK474 was used at 50 nM for *in vitro* stimulations and at 0.042 or 0.42 mg/mouse for *in vivo* experiments (Axxora, UK; L.C. Laboratories, U.S) (23-24).

DC activation and transfer

Mouse bone-marrow derived DC were generated as previously described (25). DC were pre-treated with PI3K inhibitors for 30 mins 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 DC), and 1 hour before the addition of KLH (5 µg/ml) or hs/irB16 cells (26) at a 1 to 1 ratio to DC. After washing, 5-10x10⁵ cells were injected s.c. 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). DC were also stained for expression of maturation markers using specific antibodies (MHC Class-II-FITC, CD40-FITC, CD86-A780 (all eBioscience), CD80-PE (Biolegend), CD205-Alexa 647 (AbD Serotec). Cells were analysed using Summit software on a CyanADP flow cytometer (Dako).

**Immunoblot analysis for phosphorylated PI3K**

DC 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 (2x10⁵ B16.F10 cells in C57BL/6 mice), CT26 carcinoma (5x10⁵ CT26.WT cells in BALB/c mice) or LLC (1x10⁶ LL/2 cells in C57BL/6 mice). All three tumor cell lines form solid tumors in mice when challenged s.c. with tumor cells into the flank. Tumor bearing mice were treated with s.c. injections of 5-9x10⁵ DC or with direct injections (s.c. at the site of the tumor) of flagellin (200 ng/mouse; dose
determined by titration for significant in vivo cytokine production following s.c. immunization) and/or class-I PI3K inhibitor (0.042 or 0.42mg/mouse) on days 3, 10 and 17 post tumor induction. In certain experiments, mice were injected intraperitoneally (i.p.) every 2 days from day -1 with 0.5 mg of anti-mouse IFN-γ antibody (clone XMG1.2).

T-cell responses

Popliteal lymph nodes (1x10^6/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 1-20 µg/ml of an immunodominant CD8 T-cell peptide sequence (mTRP-2^{180-188}) (Genscript) (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 post-tumor 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 performed 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 performed 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 Analysis of Variance (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 (non-normally distributed data). Statistical differences in mean values between two groups were compared using the student’s t-test. P values of less than 0.05 were considered significant.
Results

PI3K controls TLR-induced regulatory cytokine production by DC

We examined the involvement of PI3K signaling in the activation of DC with a range of TLR agonists. Stimulation of murine bone marrow-derived DC with the TLR2 agonist, zymosan, the TLR3 agonist, Poly I:C, the TLR4 agonist, 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 DC 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 DC and suppressed TLR3-induced IL-12p70 secretion (Fig. 1A). Since flagellin induced IL-12p70 and IL-10 production, the latter suppressed by the PI3K inhibitor, and since 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.

We first confirmed that DC 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 DC. Like the PI3K class I inhibitor, inhibitors of PI3Kα, β, δ and γ, significantly suppressed IL-10 secretion by flagellin-stimulated DC (Fig 1B and C). Inhibition of PI3Kβ or class-I signaling also suppressed both basal and flagellin-induced TGF-β secretion (supplementary Fig. S1B). Inhibition of PI3Kα or γ significantly suppressed IL-12 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 DC 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 DC promoted phosphorylation of PI3K p85, as demonstrated by western blot analysis with an antibody specific for phosphorylated PI3K p85 and p55 regulatory subunits (phosphorylation of p55 was not detected) (Fig. 1D). Inhibitors of PI3K β, δ, γ and class I, but not PI3K α attenuated flagellin-induced phosphorylation of PI3K p85 (Fig 1D, Supplementary Fig. S4). Expression of total p110 or p110 β or δ was not appreciably affected by any of the inhibitors examined. Although expression of total P85 was reduced by PI3K β and class I inhibitors, expression of phospho P85 relative to total P85 was reduced by inhibitors of PI3K β, δ, γ and class I (Supplementary Fig S4). These results suggest that TLR agonist-induced signaling via PI3Kβ and δ appears to be critical for the induction of IL-10 and TGF-β, but not IL-12 or maturation signals in DC.

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

Since activation of DC 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 DC should promote the induction of Th1, but not Treg cells. We tested this hypothesis using a DC adoptive transfer model, where DC were activated in vitro with flagellin and the model antigen, keyhole limpet hemocyanin (KLH), in the presence or absence of a class-I PI3K inhibitor and injected s.c. into naïve mice. Assessment of KLH-specific T cell responses in the lymph node 7 days later revealed that transfer of KLH-pulsed and flagellin-stimulated DC 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 DC 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 DC, and allowed them to selectively prime Th1 cells in vivo.

**Inhibition of PI3K β 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 DC pulsed with killed tumor cells. DC stimulated with flagellin and heat-shocked and irradiated B16 tumor cells (hs/ir B16) produced significant levels of the pro-inflammatory 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 DC 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/ir B16, whilst 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 DC (Fig. 2C).

**Inhibition of PI3K signaling in DC significantly enhances their anti-tumor 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 DC 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 DC
treated with medium only (data not shown). In contrast, treatment with DC 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 re-challenge 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 demonstrate that protection induced with DC vaccine pulsed with hs/irB16, flagellin and class-I PI3K inhibitor was associated with augmented anti-tumor effector T-cell responses in vivo.

**Direct administration of PI3K inhibitor and TLR agonist induces anti-tumor 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 Lewis lung carcinoma (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. Co-injection 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 DC (Figs 1 and 2) and demonstrates that the PI3K inhibitor has immunomodulatory effects on TLR-induced responses *in vivo*.

We next examined effect of co-administration of flagellin and PI3K inhibitor on tumor growth *in vivo* using three distinct models (B16 melanoma, CT26 carcinoma and LLC). Treatment, by s.c. administration at the tumor site, with PI3K inhibitor or flagellin alone induced a modest delay in tumor growth in all three 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 three models (Fig. 4B and C). 100% of 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 re-challenge with the tumor (Supplementary Fig. S8). These findings suggest that the combination of PI3K inhibitor and flagellin is a highly effective therapy for inducing anti-tumor immunity.

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

In order to confirm our hypothesis that direct co-treatment with PI3K inhibitor and flagellin was acting to both directly kill tumor cells and to modulate anti-tumor 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 DC 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 DC 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 \textit{in vivo} with s.c. injections of PI3K inhibitor and flagellin. When either unstimulated or FLG activated DC 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, DC that were activated \textit{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, since B16 cells treated with flagellin alone did not induce such responses (Fig. 5). Indeed tumour cells killed by PI3K inhibitor treatment were capable of inducing similar levels of IFN-γ secretion to those induced by DC presenting antigen from hs/ir B16 cells. These results support the hypothesis that inhibition of PI3K signaling is serving two discrete functions \textit{in vivo}, direct killing of tumor cells and promoting effector T-cells through modulation of TLR agonist-activated DC.

**Treatment with PI3K inhibitor and flagellin induces polyfunctional T-cells**

In order to determine if treatment with flagellin and PI3K inhibitor induced anti-tumor responses \textit{in vivo}, we assessed tumor-specific CD8$^{+}$ T-cell responses using the TRP-2 peptide (28). Spleen cells from tumor bearing mice treated with flagellin and PI3K inhibitor proliferated and secreted IFN-γ in responses TRP-2 (Fig 6A). In contrast, TRP-2-specific responses were almost undetectable in mice treated with PI3K inhibitor or flagellin alone (Fig 6A). These results indicated that treatment of mice with flagellin and PI3K inhibitor was able to induce tumor antigen-specific Th1-type responses.
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 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 FACS analysis performed 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 Fig. 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 anti-tumor 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), demonstrating that both IFN-γ and IL-17 are both required to mediate the anti-tumor 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 co-producing IFN-γ and IL-17 with no IL-10 (Fig. 7B and Supplementary Fig. S12). The frequency of IFN-γ-secreting 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-deficient (il-17⁻/⁻) mice treated with flagellin and PI3K inhibitor lacked the high proportion of IL-17 and IFN-γ co-producing 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 show 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 co-production of these two effector cytokines is required for successful tumor rejection.
Discussion

The significant new finding of this study is that a TLR agonist and PI3K inhibitor, that 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 FDA, 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 anti-tumor 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 since we have to overcome local immune tolerance which allows immune system to prevent responses against self-antigens.

The approach described in this report, 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 while 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 DC. Indeed we found that blocking PI3K signaling, especially PI3Kβ and δ, in DC inhibited IL-10 and TGF-β production and modulated T-cell
responses away from regulation and towards polyfunctional effector T-cells that were able to mediate tumor killing and rejection \textit{in vivo}.

Our findings in murine models are complemented by a recent study with human DC, 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 independent stimuli (35). This suggests that PI3K inhibitors can modulate TLR-induced cytokine production by human DC, and combined with our study, suggests that they may also enhance anti-tumor immunity in humans. When coupled with the immune stimulatory effects of TLR agonists, the overall effect of inhibiting class-I PI3K signaling directly \textit{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 DC primed \textit{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-\(\gamma\), produced by CD4, CD8 and innate immune cells plays, a major role in anti-tumor immunity (17). The role of IL-17-secreting T (Th17) cells and T-cells co-producing IL-17 and IFN-\(\gamma\) 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-\(\beta\), but not IL-1\(\beta\), IL-12p70 or IL-23 by DC and this was consistent with the induction of IFN-\(\gamma\) and/or IL-17 producing T cells \textit{in vivo}. While TGF-\(\beta\) 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 demonstrated that IL-10-defective DC pulsed with TLR agonists and hs/ir tumor cells are more protective than similarly pulsed wild-type DC (26). We have also reported that blocking TGF-β enhances Th1 and Th17 responses to infectious disease and auto-antigens (42-43). Our demonstration that IL-10+ Treg cells are reduced in vivo in mice treated with flagellin and the PI3K inhibitor, together with the finding that PI3K inhibitor suppresses IL-10 and TGF-β production by TLR5-activated DC, is consistent with this conclusion.

We found that the anti-tumor effects of co-treatment 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 co-produce IL-17 and IFN-γ mediate tumor rejection; however, we have demonstrated non-redundant 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 effector cytokines in mediating anti-tumor 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 anti-tumor effects induced our combination therapy is mediated by T-cells that secrete multiple effector cytokines,
including IFN-γ and IL-17. While IL-17 and IFN-γ co-producing cells have previously been identified, this is the first study to demonstrate 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 demonstrate that PI3K inhibitors have considerably efficacy in modulating anti-tumor immune responses induced by TLR agonists in three 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 Conflict of Interest**

Kingston Mills is a Co-founder and shareholder in Opsona Therapeutics Ltd and TriMod Therapeutics Ltd, Start-up companies involved in the development of immunotherapeutics.

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References


Figure Legends

Figure 1. Inhibition of PI3K suppresses TLR5-induced IL-10 from DC and enhances their ability to prime antigen-specific Th1 responses in vivo. A, Murine bone marrow-derived DC 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 µM). After 24 hours of culture, IL-10 and IL-12p70 concentrations were quantified in supernatant by ELISA. *** p<0.001, ** p<0.01, * p<0.05 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 DC stimulated with flagellin (FLG) in the presence and absence of specific inhibitors of PI3K α, β, δ and γ. *** p<0.001 versus untreated DC. 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+ DC expressing IL-10 or IL-12 from 4 experiments. D, Expression of PI3K p110, p110β, p110δ, p85, phosphorylated (Phos) p85 and β-actin in DC 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.

Figure 2. Inhibition of PI3K β and δ signaling modulates cytokine production by DC tumor vaccine. A, Cytokine concentration in supernatants of DC activated with flagellin and hs/irB16 tumor cells in the presence and absence of PI3K β, δ or class-I inhibitors measured by ELISA. *** p<0.001, versus hs/irB16 and flagellin. B, CD11c+ intracellular cytokine production (IL-12 and IL-10) in response to stimulation with hs/irB16, flagellin and PI3K β, δ or class-I inhibitors for 24 hours. C. Expression of activation markers (MHC Class II,
CD80 and CD86) by CD11c+ DC stimulated with hs/irB16 and flagellin +/- PI3K inhibitors. Figures on individual histograms represent the percentage of cell staining positive for each parameter compared to FMO control. Mean fluorescence intensity (MFI) values are given in parentheses.

**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 post induction with DC treated in vitro with the indicated combinations of hs/irB16, 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/ir-treated groups (Kruskal-Wallis).

**Figure 4. Direct administration of PI3K inhibitor and TLR agonist induces anti-tumor 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 post tumor induction with PBS, flagellin, class-I PI3K inhibitor or a combination of flagellin and class-I PI3K inhibitor, injected s.c 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.
Figure 5. PI3K inhibitors promote tumor-specific T-cell responses by both killing tumor cells and modulating TLR agonist induced cytokine production by DC. DC were activated with the indicated combinations of flagellin, PI3K inhibitor and B16 cells treated with heat shocking and irradiating, flagellin or flagellin with PI3K inhibitor as indicated, for 24 hours. DC were then used to stimulate splenocytes from mice treated with PI3K inhibitor and flagellin that had rejected the implanted tumors and proliferation by 3H-thymidine incorporation (A) and IFN-γ (B) and IL-10 (C) secretion quantified by ELISA.

Figure 6. Tumor-specific and polyfunctional T-cells are enhanced in mice treated with class-I PI3K inhibitor and flagellin. B16 tumor bearing mice were treated with combinations of flagellin and PI3K inhibitor as indicated on days 3, 10 and 17 post-tumor induction and sacrificed on day 22. A, Spleen cells were stimulated with TRP-2180-188 at the indicated concentrations. Proliferation (³H-thymidine incorporation) and IFN-γ and IL-10 secretion (ELISA) for 5 individual mice from each of the 4 treatment groups. Results are expressed as stimulation indeces (SI) derived from the ratio of the response for stimulated to unstimulated control cells (Range of responses: Proliferation, 1,039-7,709 cpm; IFN-γ, 92-2,141 pg/ml; IL-10, 17-392 pg/ml). Each line represents an individual mouse. * p< 0.05 versus PBS- and flagellin-treated groups  + p<0.05 versus all other groups. B, Intracellular cytokine staining and FACS analysis on tumor infiltrating lymphocytes (gated on CD3⁺CD4⁺ cells) with antibodies specific for, IL-10, IFN-γ, IL-2 and IL-17. Polyfunctional T-cell populations are plotted according to the colors indicated in the Pie slice legend with positive cells determined by comparison to FMO controls; T cells secreting combinations of detected at low frequency have been grouped and shaded grey and labeled ‘minor populations’. The
colored arcs representing the percentage of cells secreting each of the 4 individual cytokines, IFN-γ, IL-10, IL-17 or IL-2.

Figure 7. Essential roles for IFN-γ and IL-17 in the anti-tumor 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 post-tumor induction as described in figure 4. Additional groups of untreated and treated WT mice were injected i.p. 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’.
Figure 7
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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