Pharmacological Wnt ligand inhibition overcomes key tumor-mediated resistance pathways to anti-PD-1 immunotherapy

Graphical abstract

Highlights

- Proximal Wnt ligand signaling activity is associated with anti-PD-1 resistance
- Wnt signaling drives kynurenine production and PMN-MDSC accumulation in tumors
- Wnt ligand inhibition enhances the efficacy of PD-1 blockade in transgenic models
- Wnt inhibition creates a more favorable immune microenvironment in cancer patients

In brief

Anti-PD-1-refractory melanoma exhibits elevated Wnt ligand signaling activity. DeVito et al. demonstrate that pharmacologic inhibition of proximal Wnt ligand signaling sensitizes transgenic models of melanoma and lung cancer to anti-PD-1 checkpoint inhibitor immunotherapy by reversing dendritic cell tolerization and suppressing recruitment of granulocytic myeloid-derived suppressor cells to the tumor bed.

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Pharmacological Wnt ligand inhibition overcomes key tumor-mediated resistance pathways to anti-PD-1 immunotherapy

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SUMMARY

While immune checkpoint blockade is associated with prolonged responses in multiple cancers, most patients still do not benefit from this therapeutic strategy. The Wnt/β-catenin pathway is associated with diminished T cell infiltration; however, activating mutations are rare, implicating a role for autocrine/paracrine Wnt ligand-driven signaling in immune evasion. In this study, we show that proximal mediators of the Wnt signaling pathway are associated with anti-PD-1 resistance, and pharmacologic inhibition of Wnt ligand signaling supports anti-PD-1 efficacy by reversing dendritic cell tolerization and the recruitment of granulocytic myeloid-derived suppressor cells in autochthonous tumor models. We further demonstrate that the inhibition of Wnt signaling promotes the development of a tumor microenvironment that is more conducive to favorable responses to checkpoint blockade in cancer patients. These findings support a rationale for Wnt ligand-focused treatment approaches in future immunotherapy clinical trials and suggest a strategy for selecting those tumors more responsive to Wnt inhibition.

INTRODUCTION

While immune checkpoint inhibitors (ICIs) have demonstrated remarkable responses in metastatic melanoma and non-small cell lung cancer (NSCLC), most of these patients do not respond (Garon et al., 2015; Robert et al., 2015). Moreover, tumors adapt under immunotherapeutic pressure in a subset of responding patients, leading to immune escape and acquired resistance (Gide et al., 2018; Schoenfeld and Hellmann, 2020; Sharma et al., 2017). After initial clinical trial results of the anti-CTLA-4 and anti-PD-1 (aPD1) antibody combination regimen led to its use in selected cancers, other combinations with ICI have largely failed (Gide et al., 2018; Long et al., 2019; Patel and Minn, 2018). These results underscore the importance of a more sophisticated understanding of the interplay between tumor cells and the immune microenvironment that contributes to ICI resistance.

Immune exclusion has been correlated with Wnt/β-catenin pathway activation across multiple tumor types, although mutations in this pathway are rare (Luke et al., 2019; Spranger et al., 2015). This suggests that alterations in Wnt ligands, their endogenous inhibitors, as well as their receptors are likely playing an important role in driving immune evasion. Indeed, our laboratory and those of others have found that Wnt ligands, namely tumor-derived Wnt5a, attenuate the response to aPD1 therapy in mouse models and in patients with metastatic melanoma (Hugo et al., 2016; Zhao et al., 2018b). Wnt5a signals via β-catenin in tumor-associated dendritic cells (DCs), resulting in induction of indoleamine 2,3-dioxygenase (IDO1) activity, enhanced tryptophan (Trp) degradation into kynurenine (Kyn), and the generation of regulatory T cells (Tregs) (Holtzhausen et al., 2015; Zhao et al., 2018b). FoxP3+CD4+ Tregs are frequently present across cancer types and suppress anti-tumor CD8+ T cell responses to circumvent ICI efficacy (Chaudhary and Elkord, 2016; Simpson et al., 2013). Therefore, tumor-induced paracrine Wnt/β-catenin signaling promotes a program of DC tolerogenesis defined by enhanced Treg development and impaired effector T cell priming. Given the crucial role of DCs in ICI activity (Garris et al., 2018; Salmon et al., 2016; Sánchez-Paulete et al., 2016), DC tolerization as a critical ICI resistance mechanism suggests a potential strategy for the development of rational therapeutic combinations (Suryawanshi et al., 2020).

Tumors manipulate the immune microenvironment by recruiting a myriad of cell types that assist in niche formation, metastatic progression, and therapeutic resistance. This includes
myeloid-derived suppressor cells (MDSCs), which are a heterogeneous population of myeloid cells that inhibit effector CD8+ T cell activity and have been associated with impaired ICI responses (Marvel and Gabrilovich, 2015; Sade-Feldman et al., 2016; Weber et al., 2016; Weber et al., 2018). Granulocytic MDSCs (polymorphonuclear [PMN]-MDSCs) are recruited to the tumor bed through the expression of CXCR2 ligands, including CXCL2 and CXCL5 (Forsthuber et al., 2019; Highfill et al., 2014; Steele et al., 2016). Our group has found that Wnt5a promotes chemokine-driven recruitment of PMN-MDSCs through autocrine Yap signaling in the tumor, resulting in the suppression of infiltrating activated CD8+ T cells, disease progression, and aPD1 resistance (Theivanthiran et al., 2020). Altogether, these studies implicate tumor-derived Wnt ligand signaling in a broad reprogramming of the immune microenvironment and as a node for both intrinsic and adaptive resistance to immunotherapy. Given these findings, we sought to evaluate the efficacy of several Wnt ligand inhibition strategies in the context of ICI therapy.

Inhibition of the Wnt/β-catenin pathway to date has involved either enhancing β-catenin degradation or blocking its interaction with target genes, impeding the release of Wnt ligands through inhibition of porcupine (PORCN), or by interfering with Wnt ligand-Fz receptors (Jung and Park, 2020). Our group has found that Wnt5a promotes chemokine-driven recruitment of PMN-MDSCs through autocrine Yap signaling in the tumor, resulting in the suppression of infiltrating activated CD8+ T cells, disease progression, and aPD1 resistance (Theivanthiran et al., 2020). Altogether, these studies implicate tumor-derived Wnt ligand signaling in a broad reprogramming of the immune microenvironment and as a node for both intrinsic and adaptive resistance to immunotherapy. Given these findings, we sought to evaluate the efficacy of several Wnt ligand inhibition strategies in the context of ICI therapy.

Inhibition of the Wnt/β-catenin pathway to date has involved either enhancing β-catenin degradation or blocking its interaction with target genes, impeding the release of Wnt ligands through inhibition of porcupine (PORCN), or by interfering with Wnt ligand-Fz receptors (Jung and Park, 2020). Given the lack of activating β-catenin pathway mutations, we focused on more proximal pharmacologic approaches that targeted Wnt ligands and their receptors. OMP-18R5 (vantictumab) is a monoclonal antibody (mAb) that binds to Fzd receptors 1, 2, 5, 7, and 8, which has demonstrated safety in phase I studies (Davis et al., 2020). OMP-54F28 (ipafricept), a recombinant fusion protein of Fzd8 and the immunoglobulin (lg)G1 Fc domain, also interferes with Wnt ligand signaling by acting as an extracellular trap and has proven safety in phase I trials (Moore et al., 2019). Other strategies involve the blockade of PORCN, which catalyzes the palmitoylation of Wnt proteins, which is necessary for both their secretion as well as their binding to Fzd receptors (Figure 1A). There are several ongoing trials in various combinations with small-molecule PORCN inhibitors including LGK974 (WNT974), RXC004, CGX1321, and ETC-159 (ETC-1922159) (Zhong et al., 2019). Studies to date, however, have focused on targeting tumor-dependent Wnt/β-catenin signaling for chemotherapy resistance rather than modulation of the tumor immune response (Jung and Park, 2020).

Using autochthonous mouse models to better recapitulate the immune tolerization mechanisms observed in human cancers, we characterize the anti-tumor activity and immunological impact of Wnt ligand inhibition in combination with aPD1 in melanoma and NSCLC. This work describes the underlying immunologic mechanisms of these agents within the tumor microenvironment and compares their efficacy with selective IDO1 inhibition. In addition, we explore how the expression of components of the Wnt ligand/Fzd receptor signaling axis correlates with ICI resistance in human melanoma, and we further analyze the immunologic impact of Wnt ligand inhibition in cancer patients.

RESULTS

Wnt ligand signaling influences responses to checkpoint inhibitor therapy

Prior studies have shown an association between Wnt/β-catenin activation and T cell exclusion; however, only ~15% of T cell-poor tumors harbor genetic mutations or copy number variations impacting downstream mediators that drive the activation of the
\(\beta\)-catenin signaling pathway (Luke et al., 2019; Spranger et al., 2015). This suggests that paracrine and autocrine Wnt signaling plays an important role in suppressing anti-tumor immunity (Figure 1A). This is consistent with transcriptional analyses demonstrating that escape from aPD1 immunotherapy in an autochthonous model of BRAFV600EPTEN\(^{-/-}\) (BP) melanoma is associated with enhanced Wnt pathway activation, including elevated expression of several Wnt ligands, Wnt receptors, as well as both canonical and non-canonical targets (Figure 1B).

To examine the role of paracrine and autocrine Wnt ligand signaling in aPD1 resistance in melanoma patients, we first reanalyzed a previously published RNA sequencing (RNA-seq) data set of patients with metastatic melanoma who had undergone treatment with aPD1 (GEO: GSE78220) (Hugo et al., 2016). This work revealed several Wnt ligands and Fzd receptors to be upregulated in non-responders relative to responders, while the RNF43 E3 ubiquitin ligase, a negative regulator of surface Fzd receptor expression, was reciprocally upregulated in responders (Figure 1C). To expand on this analysis, we used a NanoString-based approach to explore the differential expression of Wnt signaling components in aPD1 responding versus non-responding melanoma patients. These data confirmed evidence that both canonical and non-canonical Wnt signaling is elevated in aPD1-refractory melanomas (Figure 1D; Table S2). In addition to an upregulation of Fzd receptors, Wnt ligands, and the co-receptor LRPR, expression of both PYGO1 and DVL1, which are known to potentiate Wnt signaling, were also more prominent in non-responding patients (Clevers and Nusse, 2012; Katoh and Katoh, 2007; Mieszczanek et al., 2008) (Figures 1A and 1D). We also observed a significant elevation in DKK2 and SFRP2, which are positively upregulated by the Wnt signaling pathway (Lescher et al., 1998; Li et al., 2005; Xiao et al., 2018). Overall, these data provide supportive evidence that tumors from aPD1 non-responders exhibit enhanced Wnt signaling activity. Notably, this work further showed an expected increase in CD8A, GZMB, and IDO1 expression in aPD1 responders. IDO1 has been previously described to be upregulated in responders to ICI therapy, likely in response to interferon (IFN)-\(\gamma\) signaling (Gide et al., 2018; Hamid et al., 2011; Yoshida et al., 1991). Importantly, these results in human melanoma are consistent with the murine transcriptional data, highlighting the translatability of the autochthonous BP melanoma model for further studies.

Wnt ligand inhibition suppresses DC-mediated Treg generation

Given recent studies identifying the DC as a critical mediator of immunologic responses to aPD1 (Salmon et al., 2016; Spranger et al., 2017), and our previous data implicating Wnt5a in DC-mediated Treg differentiation (Holtzhausen et al., 2015; Zhao et al., 2018b), we set out to develop a pharmacologic strategy to reverse Wnt ligand-mediated DC tolerization (Figure 1A). The Fzd receptor antagonistic antibody OMP-18R5 and the Wnt ligand trap OMP-54F28 both inhibit Wnt ligand induction of \(\beta\)-catenin activation in 293T-TCF/LEF luciferase reporter assays (Figures 2A and S1A) (Fischer et al., 2017; Gurney et al., 2012; Jimeno et al., 2017; Moore et al., 2019). Similarly, these inhibitors suppressed \(\beta\)-catenin levels in transgenic melanomas in situ (Figure S1B). Further work showed these agents also inhibit Wnt-induced IDO1 expression by DCs and reverse Wnt5a-induced DC-mediated Treg generation in vitro (Figures 2B, 2C, and S1C). This is consistent with our previous studies demonstrating that the PORCN inhibitor, CS9, blocks both the secretion of the Wnt5a ligand as well as melanoma-mediated up-regulation of IDO1 expression by DCs (Holtzhausen et al., 2015) (Figure 1A). In a similar fashion to OMP-18R5, treatment of a melanoma cell line derived from the autochthonous BP model with CS9 reverses tumor conditioned media (CM)-induced DC-dependent Treg generation (Figure 2D).

Wnt ligand inhibition enhances aPD1 therapy in an autochthonous model of melanoma

To further determine the effects of pharmacologic Wnt ligand inhibition in vivo and evaluate synergy with aPD1 antibody immunotherapy, we performed additional experiments utilizing the syngeneic BP melanoma model. Both OMP-54F28 and OMP-18R5 in combination with aPD1 were found to suppress primary tumor growth over aPD1 alone (Figure 2E). In addition, Wnt ligand inhibition in combination with aPD1 eliminated metastatic progression to the lung based on hematoxylin and eosin (H&E) staining (Figures 2F and S1D). Further immunohistochemistry (IHC) studies showed that both Wnt ligand inhibitors enhanced tumor CD8\(^{+}\) T cell infiltration, although OMP-18R5 generated the most robust anti-tumor immune response (Figures 2G and S1E). This is consistent with an increase in the tumor-infiltrating CD8\(^{+}\) T cell/Treg ratio following OMP-18R5/aPD1 combination therapy based on flow cytometry (Figure 2H).

We have previously demonstrated that the autochthonous BP melanoma model exhibits a modest response to aPD1 immunotherapy followed by disease progression despite continued PD-1 blockade (Zhao et al., 2018a). In this model, combination OMP-18R5/aPD1 effectively impedes primary melanoma progression compared to aPD1 alone, while additional H&E studies also demonstrated a suppression in lung metastasis (Figures 3A, 3B, and S2A). Importantly, this anti-tumor effect correlated with enhanced tumor-infiltrating CD8\(^{+}\) T cell/Treg ratios as well as increased numbers of infiltrating CD8\(^{+}\) cells specific to the melanoma-associated antigen, tyrosinase-related protein-2 (TRP2), based on IFN-\(\gamma\) enzyme-linked immunospot (ELISpot) assays (Figures 3C, 3D, S2B, and S2C). A more modest anti-tumor immune effect was observed in the same tumor model with OMP-54F28 (Figure S3), which led us to primarily pursue the analysis of OMP-18R5 in subsequent experiments.

The concept of pharmacologic Wnt signaling inhibition was initially developed to limit stem cell longevity and therefore inhibit tumor progression (Krishnamurthy and Kurzrock, 2018).

To determine whether Wnt inhibition impacted tumor cell proliferation in our model, we conducted in vitro studies showing that neither OMP-18R5 nor OMP-54F28 affected BP melanoma cell proliferation (Figure S2D). To further investigate whether the effects of Wnt ligand inhibition in the BP transgenic melanoma model is dependent on the host immune system, we repeated the above in vivo experiment following antibody-directed ablation of CD8\(^{+}\) T cells (Figure S2E). Importantly, this study demonstrated that the ablation of effector CD8\(^{+}\) T cells eliminated the therapeutic effect of OMP-18R5, indicating that Wnt inhibition...
suppresses primary and metastatic progression via a cytotoxic T cell-mediated mechanism (Figures 3E, S2E, and S2F).

Our previous data suggest that aPD1-refractory melanomas may be more sensitive to Wnt ligand inhibition (Figures 1B–1D). To test this hypothesis, we repeated the above experiment in the BP transgenic melanoma model but delayed the introduction of a small molecule PORCN inhibitor, ETC-159, to block Wnt ligand secretion until approximately day 14 of treatment with aPD1, allowing sufficient time for the development of ICI resistance (Zhao et al., 2018a). Consistent with our prior observations that aPD1 resistance is associated with enhanced Wnt ligand signaling (Figures 1B–1D), the introduction of the ETC-159 PORCN inhibitor stabilized primary tumor progression in the autochthonous melanoma model (Figure 3F). These effects correlated with a significant increase in tumor-infiltrating CD8+ T cells and a diminished number of tumor-resident Tregs (Figure 3G). We also observed ETC-159 to correspondingly inhibit the enhanced β-catenin signaling observed during aPD1 therapy in tumor-associated DC populations (Figure 3H) as well as in the tumor tissues themselves (Figure 3I). Overall, these studies suggest that Wnt ligand inhibition augments aPD1 efficacy in a manner at least partially dependent on the host immune system.

Wnt ligand inhibition enhances aPD1 therapy in an autochthonous model of NSCLC

To determine whether the immunologic impact of Wnt inhibition exists beyond melanoma, we turned our attention toward additional studies focused on NSCLC. Relative to melanoma, the
response rates to aPD1 in NSCLC patients have been more modest (Reck et al., 2016). As a result, there is an urgent need for strategies to enhance the efficacy of ICI therapy in NSCLC. Initially, we performed a similarly designed experiment using the syngeneic Lewis lung carcinoma (LLC) model. We observed OMP-18R5/aPD1 combination therapy to modestly suppress primary tumor progression while significantly enhancing numbers of tumor-infiltrating CD8+ T cells, including an increased population of CD8+ T cells capable of recognizing the LLC-associated tumor antigen MUT1 (Mandelboim et al., 1994).

To test whether these effects could further extend to an autochthonous model of NSCLC, we initiated studies using the KrasG12Dp53flox/flox (KP) NSCLC system that can be induced...
via the intra-nasal delivery of a Cre recombinase-expressing adenovirus (Ad-Cre) (DuPage et al., 2009). Since tumors from these mice have been shown to contain a Wnt-producing niche, we hypothesized that Wnt ligands could be driving a similar aPD1 resistance program as in melanoma (Tammela et al., 2017). Approximately 8–10 weeks following Ad-Cre delivery, mice underwent baseline micro-computed tomography (micro-CT) imaging followed by therapy. Serial micro-CT imaging and tumor volume contouring were utilized at weeks 14 and 17 to monitor primary tumor progression in the lung in each treatment group. This work demonstrated the OMP-18R5/aPD1 combination regimen to be superior to aPD1 mAb monotherapy (Figures 4A, 4B, S4D, and S4E). This imaging data correlated well with diminished lung weights in the OMP-18R5/aPD1 combination group and was further supported by a reduced pulmonary tumor burden based on H&E microscopy (Figures 4C and 4D).

Figure 4. Immunotherapeutic properties of OMP-18R5 extend to an autochthonous model of NSCLC

(A) Cre-expressing adenoviral vector was administered intra-nasally to autochthonous KP mice, inducing primary lung tumor development. At 10 weeks, mice were initiated on IgG ctrl, aPD1, OMP-18R5, or OMP-18R5/aPD1 (n = 6/group). All associated data are representative of two independent experiments.

(B) Micro-CT quantification of tumor volume at weeks 10, 14, and 17 in (A). (Left) Final tumor volumes. Each point represents an individual lung tumor. (Right) Representative images from each treatment group. Red arrows indicate lung tumors. Statistical analyses for (A) and (B) are based on one-way ANOVA followed by a Tukey post hoc test.

(C) Lung weights related to (A). Weights are normalized to lungs harvested from age-matched non-tumor-bearing mice.

(D) Representative H&E sections from each treatment group taken at week 17. Scale bars, 2,000 μm.

(E) IHC quantification of intra-tumoral CD8⁺ T cells in the experiment described in (A) (n = 5/group).

(F) Quantification of KP cell line-derived antigen-specific splenic CD8⁺ T cells harvested in (A) based on IFN-γ ELISpot (n = 3–5/group).

(G) Kaplan-Meier survival analysis of mice related to (A). Statistical analysis based on log-rank test, p = 0.06. A Student’s two-tailed t test was performed when comparing aPD1 monotherapy groups versus combination therapy. All data show mean ± SEM. **p < 0.01, *p < 0.05. See also Figure S4.
Anti-tumor responses to the OMP-18R5/aPD1 combination regimen further correlated with enhanced CD8+ T cell infiltration into tumor tissues as well as with the development of enhanced numbers of tumor antigen-specific CD8+ T cells (Figures 4E, 4F, S4F, and S4G). Importantly, all of these findings were in line with improved survival of the KP mice in the combination treatment group over aPD1 monotherapy (Figure 4G). These data further indicate that Wnt ligand inhibition promotes the generation of an effective anti-tumor immune response in multiple tumor histologies and supports the therapeutic relevance of targeting Wnt ligand signaling.

**Wnt ligand inhibition diminishes local Kyn levels in the tumor microenvironment**

The immunoregulatory enzyme IDO1 has been shown to play an important role in regulating anti-tumor immunity by suppressing effector CD8+ T cell expansion while driving Treg differentiation through the production of the metabolite Kyn (Munn and Mellor, 2007; Munn and Mellor, 2016). While extensive literature has described the critical role of IDO1 in the regulation of anti-tumor immunity in both pre-clinical and early clinical studies, a recent phase III clinical trial (KEYNOTE-252/ECHO-301) showed that the selective IDO1 inhibitor epacadostat in combination with pembrolizumab failed to improve clinical outcomes for metastatic melanoma patients relative to pembrolizumab alone (Long et al., 2019). These disappointing results have been hypothesized to be due, at least in part, to compensatory Trp degrading enzymes that also contribute to Kyn levels such as TDO2 (Muller et al., 2019). We have previously demonstrated that Wnt5a ligand signaling in local DC populations within the tumor microenvironment drives the expression of IDO1 (Holtzhausen et al., 2015). Further studies have shown this same Wnt-dependent pathway to promote the synthesis of protoporphyrin IX (PpIX), a heme-derived compound, which serves as the ratelimiting prosthetic group for both IDO1 and TDO2 (Lewis-Ballester et al., 2016; Zhao et al., 2018b). Based on these data, we hypothesized that the inhibition of paracrine Wnt ligand signaling could result in potent suppression of Kyn production by DCs in the tumor and lymph node (LN) microenvironments (Metz et al., 2010) (Figure 5A). Initial studies found that Wnt ligand blockade suppresses the synthesis of PpIX in DCs by flow cytometry (Zhao et al., 2018b) (Figure 5B). These results demonstrated this pharmacologic strategy to regulate the activity of other Trp degrading enzymes beyond IDO1 in DCs. Notably, we found no evidence that this pathway regulates tumor-dependent expression of either IDO1 or TDO2 or DC-dependent expression of TDO2 (Figures S5A and S5B). In addition, tumor cell PpIX levels were not impacted by Wnt5a stimulation, suggesting that the Wnt ligand effect on this pathway is restricted to DCs and perhaps other antigen-presenting cells (APCs) in the tumor microenvironment (Figure S5C) (Zhao et al., 2018b).

To test whether Wnt ligand inhibition effectively suppresses Kyn generation and subsequent Treg differentiation in vivo, we performed a multi-arm experiment utilizing the autochthonous BP melanoma model to compare the ability of epacadostat versus OMP-18R5 to enhance the efficacy of aPD1. This work demonstrated OMP-18R5 to reproducibly suppress melanoma progression more effectively than epacadostat when combined with aPD1 (Figure 5C). Combination OMP-18R5/aPD1 therapy also enhanced the generation of TRP2-specific CD8+ T cells based on IFN-γ ELISpot analysis compared to epacadostat/aPD1 (Figures 5D and S5D). To determine whether the therapeutic effect observed by OMP-18R5 is associated with its ability to suppress Kyn levels in situ, we harvested both tumor and LN tissues from melanoma-bearing mice undergoing treatment with either epacadostat or OMP-18R5 for analysis by a Kyn-specific enzyme-linked immunosorbent assay (ELISA) and liquid chromatography-tandem mass spectrometry (LC-MS/MS). As expected, diminished levels of Kyn were observed in both tumor and tumor-draining LN (TDLN) tissues following treatment with epacadostat, while these levels trended lower in those tissues exposed to OMP-18R5 (Figures 5E, S5E, and S5F). Consistent with these findings, we identified a more substantial suppression in tumor-infiltrating Treg populations following OMP-18R5/aPD1 over epacadostat/aPD1 (Figure 5F). Based on our data suggesting that Wnt signaling activity is enhanced in the aPD1-refractory setting (Figures 1 and 3H), we further postulated that the inhibition of Wnt ligand signaling would be superior to selective IDO1 inhibitor therapy in aPD1-resistant tumors. We therefore compared delayed epacadostat to delayed ETC-159 after prior exposure to aPD1 mAb in the autochthonous BP melanoma model. Indeed, this study also found ETC-159 to impair tumor growth more effectively than epacadostat following progression through continuous aPD1 therapy (Figure 5G).

Altogether, these data suggest that Wnt ligand inhibition enhances anti-tumor immunity at least partially by suppressing the production of Kyn in the tumor and TDLN microenvironment. These studies provide support for pharmacologically targeting the upstream regulatory pathways common to all Trp-degrading enzymes as a strategy for suppressing Kyn generation and Treg development. However, reconciling the relative levels of Kyn following Wnt ligand inhibition versus IDO1 inhibition with the superior anti-tumor response observed with Wnt targeted therapy, we hypothesized that the immunological impact of Wnt inhibition may extend beyond the production of Kyn. We therefore initiated studies to investigate alternative mechanisms by which Wnt ligand blockade may support responses to checkpoint inhibitor immunotherapy.

**Wnt ligand inhibition suppresses PMN-MDSC recruitment to the tumor bed**

We have found that a noncanonical Wnt ligand-YAP signaling axis plays an important role in regulating the recruitment of PMN-MDSCs to the tumor bed by promoting the expression of CXCR2-dependent chemokines in response to PD-1 blockade (Theivanthiran et al., 2020). Notably, this study found the CXCL5 ligand signaling to be most responsive to aPD1 therapy as well as to Wnt5a stimulation and demonstrated the recruitment of this PMN-MDSC population to suppress CD8+ T cell responses and promote tumor progression. We therefore hypothesized that Wnt ligand inhibition would inhibit CXCL5 chemokine expression and suppress PMN-MDSC accumulation in tumors. Indeed, we found OMP-18R5 to inhibit Wnt5a-dependent CXCL5 expression and YAP1 stabilization by BP melanoma cells in vitro, as well as CXCL5 expression by BP melanomas in vivo (Figures 6A–6C). Similarly, ETC-159 suppressed aPD1-induced CXCL5 and
CXCL2 expression by BP melanomas in vivo based on qRT-PCR analysis (Figures 6D and S6A). Consistent with our prior studies, we found that tumor-associated PMN-MDSCs increase in response to aPD1, but this effect diminished with the addition of either ETC-159 or OMP-18R5 (Figures 6E, 6F, and S6B) (Theivanthiran et al., 2020). Furthermore, Wnt inhibitor-mediated suppression of PMN-MDSC recruitment is independent of the tumor model since we observed a similar inhibition of PMN-MDSC recruitment to LLC tumors following OMP-18R5 treatment (Figure 6G).

These data suggest that Wnt ligand inhibition can generate an immune microenvironment that is more conducive to the development of anti-tumor immunity via multiple mechanisms. In light of prior studies highlighting a negative relationship between circulating levels of PMN-MDSCs and the efficacy of aPD1, these data suggest that Wnt ligand inhibition is expected to augment responses to checkpoint inhibitor immunotherapy by also blocking PMN-MDSC recruitment to the tumor bed (Meyer et al., 2014; Weber et al., 2016).

Wnt ligand inhibition promotes the development of an immune microenvironment that is more conducive to checkpoint inhibitor responses in cancer patients During a phase I all-solid tumor dose-escalation study conducted with the ETC-159 PORCN inhibitor, a patient with colorectal
cancer (CRC) with RSPO fusion (patient #1) and a patient with presumed cholangiocarcinoma (patient #2) each underwent both pre- and post-treatment tissue biopsies, allowing for differential gene expression studies using the NanoString transcriptional analysis platform (Figure 7A; Table S3). Overlapping increases in several genes associated with enhanced immunogenicity, including **CD8A**, **CD27**, **CD40**, **IFNG**, **PDCD1** (PD-1), and various granzyme isoforms were observed in both patients as well as **CD86** and **PRF1** in patient #2. In addition, multiple genes associated with immunosuppression, including **ARG1** and **ARG2**, the MDSC recruiting chemokines **CXCL1/CXCL2** and **IL10**, and the Treg markers **FOXP3** and **NRP1** (neuropilin) were downregulated with ETC-159 treatment (Figure 7B; Table S4). Finally, both treated tumors exhibited decreased **TCF7** and **CCND3** expression, verifying downstream inhibition of the Wnt/β-catenin pathway (Figure S6C).

Overall, these results support our pre-clinical data and indicate that Wnt ligand blockade represents a promising strategy for converting the tumor microenvironment into a more favorable state for the generation of an effective anti-tumor immune response to aPD1.

**Wnt ligand inhibition does not significantly impact tumor DC recruitment**

Previous studies have shown that stabilization (STA) of β-catenin in a related BRAF^V600E/Pten−/−/Bcat^STA melanoma model suppresses tumor CCL4 expression, reducing antigen cross-presenting CD103^+ CD11c^+ Tumor Cell infiltration (Damsky et al., 2011; Spranger et al., 2015). While we observed a modest increase in the expression of DC-associated genes, **ITGAX** and **FLT3L**, in response to Wnt ligand inhibition in patient #1 (Figure 7B; Table S4), these changes were not observed in patient #2 despite a significant increase in cytolytic T cell markers in the treated tumor. Moreover, we did not appreciate an increase in the
tumor-infiltrating CD103+ subset of MHC class II CD11c+ DCs with combination aPD1 and OMP-18R5 (Figure S7A, left) or ETC-159 in the BP melanoma model (Figure S7A, right). We also did not see meaningful alterations in Ccl4 expression in murine tumors treated with OMP-18R5 or ETC-159 and did not observe differences in CCL4 or ATF3 expression levels between aPD1 responder and non-responder melanoma patients in the Hugo et al. (2016) human RNA-seq dataset (Figures S7B and S7C). Larger tissue-based studies will be necessary to determine the relative role of DC recruitment on the observed immunological impact of pharmacologic Wnt pathway inhibition.

**DISCUSSION**

The Wnt/\(\beta\)-catenin signaling pathway has been associated with tumor immune evasion as well as ICI resistance in mouse models and patients (Holtzhausen et al., 2015; Luke et al., 2019; Spranger et al., 2015; Theivanthiran et al., 2020). Research thus far suggests that this process occurs through at least three mechanisms: paracrine Wnt ligand-induced DC tolerance (Holtzhausen et al., 2015; Hong et al., 2015a; Manicassamy et al., 2010; Zhao et al., 2018b), autocrine Wnt ligand-driven PMN-MDSC recruitment (Theivanthiran et al., 2020), and tumor-intrinsic \(\beta\)-catenin signaling, leading to a suppression of CCL4 and a reduction in the influx of CD103+ antigen cross-presenting DCs into the tumor bed (Spranger et al., 2015).

The observation that only a limited percentage of T cell-poor tumors harbor downstream activating mutations that drive the \(\beta\)-catenin signaling pathway strongly suggests that regulators of paracrine and autocrine Wnt ligand activity contribute to immune exclusion (Luke et al., 2019; Spranger et al., 2015). Indeed, mutations promoting activation of the Wnt/\(\beta\)-catenin pathway in CRCs are not associated with differences in immune cell infiltration, further supporting this concept (Mlecnik et al., 2016). Additionally, our previous data indicate that a non-canonical YAP-dependent pathway in tumors is responsible for promoting PMN-MDSC recruitment in response to Wnt5a, implying that alterations impacting upstream paracrine/autocrine Wnt ligand signaling are likely to play an important role beyond downstream mutations exclusively activating the canonical Wnt/\(\beta\)-catenin signaling pathway. This is consistent with our finding of increased expression levels of SFRP2 in aPD1-resistant melanomas, which has been shown to promote a shift toward non-canonical pathway activation (Brinkmann et al., 2016; Sun et al., 2016). These findings led us to investigate whether modulating upstream paracrine/autocrine Wnt ligand signaling could influence aPD1 outcomes.

We observed that pharmacologic inhibition of Wnt ligand-receptor interactions enhanced aPD1 in both syngeneic and autochthonous models of melanoma. This was attributable to the effect of Wnt ligand inhibition on the immune system, as...
this strategy does not affect tumor cell proliferation in vitro and requires CD8+ T cells for activity in vivo. This is further supported by studies showing Wnt ligand inhibition to promote tumor antigen-specific CD8+ T cell responses while reducing tumor-infiltrating Treg and PMN-MDSC populations within tumors. Indeed, this is also consistent with our finding that treatment of cancer patients with ETC-159 alters the tumor immune microenvironment in a manner that is expected to be more favorable for generating responses to checkpoint inhibitor therapy.

To further investigate activity outside of melanoma, we also examined the immunologic impact of Wnt ligand inhibition in a LLC model and a KP autochthonous model of NSCLC. Initial studies showed Wnt ligand inhibition to sensitize LLC tumors to ICI therapy. Contrary to previous observations showing a lack of response to dual immune checkpoint blockade in the autochthonous NSCLC model (Pfirschke et al., 2016), additional studies demonstrated that Wnt ligand inhibitor/aPD1 combination therapy enhanced tumor antigen-specific immunity while suppressing tumor progression and extending survival. Overall, we demonstrate that Wnt ligand inhibition improves aPD1 responses in two clinically relevant autochthonous mouse models of melanoma and NSCLC.

DCs direct the cellular cytotoxic immune response and are necessary for successful immune checkpoint blockade (Garris et al., 2018). Previously, we associated the Wnt/β-catenin cascade with DC tolerance through induction of IDO1 expression and enzymatic activity, culminating in Treg generation via Kyn production (Zhao et al., 2018b). In the present study, we found that Wnt ligand inhibition reversed β-catenin signaling in tumor-draining LN DCs while inhibiting tryptophan-degrading enzymatic activity both in vitro and in vivo. These data were consistent with the additional finding that Wnt ligand inhibition suppressed DC PpIX synthesis (Zhao et al., 2018b). While DC-mediated Treg generation was induced by Wnt ligand stimulation or tumor-conditioned media, as we have observed previously (Holtzhausen et al., 2015), this effect could be reversed by Fzd receptor blockade and PORCN inhibition, respectively. These results are consistent with data generated by others showing that targeting the Wnt/β-catenin pathway via DC-specific genetic deletion of LRP co-receptors can enhance anti-tumor immunity (Hong et al., 2015b; Manicassamy et al., 2010). These results are further in line with recent studies showing ETC-159 to improve CD8+ T cell/Treg ratios in a humanized mouse model of microsatellite stable CRC (Bagby et al., 2020).

Given that others have demonstrated the Wnt/β-catenin pathway to suppress DC recruitment through CCL4 repression, we evaluated CD103+ DC accumulation in the tumor bed and alterations in CCL4 in the tumor microenvironment in response to Wnt ligand inhibition. We did not observe significant differences by either of these parameters to sufficiently account for the mechanism of action of Wnt inhibition (Figure S7). Opposed to our studies that used a BP autochthonous melanoma model treated with pharmacologic inhibitors to the Wnt ligand signaling pathway, Spranger et al. (2015) utilized a constitutively active β-catenin derivative of the BP melanoma model. The discrepancies in these results may be associated with the relative differences in potency between genetic activation and the pharmacologic inhibition of the β-catenin pathway. However, the differential impact of these models on non-canonical Wnt signaling may also be a contributing factor.

Significant scientific effort has been invested in developing methods of inhibiting Trp catabolizing enzymes (Hou et al., 2007; Muller et al., 2005; Munn, 2012). The selective IDO1 inhibitor epacadostat showed promise in preclinical studies (Prendergast et al., 2017); however, it did not achieve any meaningful activity in a recent phase III clinical trial at the selected dose (Long et al., 2019). Our data indicate that targeting Wnt ligands, which are upstream regulators of IDO1 and TDO2 activity, can outperform selective IDO1 inhibition in the generation of antigen-specific T cell responses as well as the control of primary tumor growth at least, in part, by suppressing Kyn production and Treg differentiation. In addition to supporting the immunologic significance of blocking Wnt ligand signaling, these data further suggest that a Wnt ligand genetic signature may serve as a predictive biomarker for targeted IDO1/TDO2 inhibitors (Holtzhausen et al., 2015). A previous study has shown that tumor-expressed IDO1 promotes the recruitment of MDSCs via Tregs using an IDO1-overexpressing B16 model (Holmgaard et al., 2015). We did not find differences in intratumoral MDSCs with epacadostat treatment in our system. Moreover, our results and previously published observations (Theivanthiran et al., 2020) point toward an autocrine Wnt ligand-induced upregulation of PMN-MDSC recruiting chemokines. This is further supported by our observations that OMP-18R5 impairs Wnt ligand-induced CXCL5 production in our melanoma cell line in vitro and the autochthonous melanoma model in vivo. We have thus expanded upon our previous findings where autocrine tumor Wnt ligand stimulation supports PMN-MDSC recruitment during aPD1 treatment by demonstrating that this process is reversible with various Wnt ligand inhibition strategies. Notably, we have not observed any alterations in monocyte or macrophage populations with Wnt ligand inhibitors, suggesting that the effect of Wnt ligand inhibition may be restricted to PMN-MDSCs. While others have observed Wnt pathway activation in association with resistance to immunotherapy, a higher resolution analysis of the Wnt ligand/Fzd receptor signaling axis as a driver of aPD1 resistance has not previously been conducted (Gide et al., 2018).

A relevant concern associated with targeting the Wnt/β-catenin pathway clinically is adverse effects (AEs), which could hinder treatment efficacy and feasibility, particularly in combination with other agents. A phase I study of patients treated with single-agent PORCN inhibitor LGK974 (WNT974) showed grade 3/4 AEs including asthenia and fatigue in 2% of patients (Janku et al., 2015). Phase I data on OMP-18R5 reported grade 3/4 drug-related AEs, including nausea, fatigue, and dysgeusia in 29% of patients, while OMP-54F28 has been associated with grade 3 AEs, including neutropenia in 21.6% of patients (Davis et al., 2020; Moore et al., 2019). Bone-related AEs, including bone fracture, emerged in the initial part of both phase I studies and were not observed subsequently after monitoring protocols, intermittent dosing, and prophylactic bone protective agents were initiated (Davis et al., 2020; Moore et al., 2019). This safety profile has been manageable and lacks immune-related AEs, an encouraging attribute for combining these agents with ICI therapy (Janku et al., 2020).
This current study has important limitations to be considered. Fzd receptor blockade may differ in its mechanism of action from the ablation of all soluble Wnt ligands. Although we did not compare OMP-18R5 directly to ETC-159, the in vitro and in vivo activities associated with these agents were consistently similar. Given our previous data, we did not extensively examine the potential effects of Wnt inhibition on cell types other than tumor cells, PMN-MDSCs, and DCs. Further investigation is warranted into the effect that this treatment strategy may have on the diverse tumor microenvironment, particularly in patients as Wnt inhibition progresses into clinical trials with ICI therapies. Finally, the clinical specimen-derived data presented herein are based on a limited number of samples. Additional correlative studies are needed to expand these studies to a larger array of tumor types in a larger cohort of patients.

In summary, we demonstrate that Wnt ligand inhibition enhances aPD1 therapy and rescues tumors progressing through aPD1 in autochthonous mouse models that recapitulate human melanoma and NSCLC. By attenuating tumor-induced DC tolerance and PMN-MDSC recruitment, inhibiting Wnt ligand signaling affects critical nodes in tumor immune escape and addresses key drivers of immunotherapy resistance. We further provide correlative data derived from clinical tumor specimens, indicating that Wnt ligand inhibition can support cytolytic T cell activity in human tumors. PORCN inhibitors, including ETC-159, are currently being investigated in combination with aPD1 mAb therapies in early phase clinical trials. Our preclinical and correlative clinical data can inform treatment sequencing as well as biomarker development for future trials investigating this strategy. Importantly, these data suggest that tumors exhibiting elevated levels of paracrine/autocrine Wnt signaling activity based on gene expression profiling studies would be expected to respond more favorably to combination aPD1/Wnt ligand inhibitor therapy over aPD1 monotherapy. Cumulatively, our work demonstrates that pharmacologic inhibition of Wnt ligands represents a promising approach for reversing tumor-mediated immune tolerance and enhancing the efficacy of PD-1 blockade.

**STAR METHODS**

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**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2021.109071.

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**AUTHOR CONTRIBUTIONS**

B.A.H., A.H., and J.H.S. conceptualized the project. B.A.H. and N.C.D. designed all experiments. B.A.H., N.C.D., and M.S. analyzed all data. M.S. and C.X. provided technical support. A.K.S.S., G.M.B., and V.N.-D. provided clinical resources for the project. B.A.H. and N.C.D. supervised all experiments. B.A.H., N.C.D., and M.S. wrote the manuscript. B.A.H., N.C.D., A.H., M.S., and M.P.P. reviewed and edited the manuscript.

**DECLARATION OF INTERESTS**

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Anti-mouse CD45, PerCp-Cy5.5 conjugated, clone:30-F11 | BD PharMingen | Cat# 550994; RRID:AB_394003 |
| Anti-mouse CD3ε, FITC conjugated, clone: 145-2C11 | BD PharMingen | Cat# 553061; RRID:AB_394594 |
| Anti-mouse CD8α, BV510 conjugated, clone: 53-6.7 | BD PharMingen | Cat# 563068; RRID:AB_2687548 |
| Anti-mouse CD4, APC conjugated, clone: RM4-5 | BD PharMingen | Cat# 553051; RRID:AB_398528 |
| Anti-mouse Foxp3, PE conjugated, clone: MF23 | BD PharMingen | Cat# 560408; RRID:AB_1645251 |
| Anti-mouse CD11b, PE conjugated, clone: M1/70 | BD PharMingen | Cat# 557397; RRID:AB_396680 |
| Anti-mouse F4/80, APC conjugated, clone: BM8 | BioLegend | Cat # 123116; RRID:AB_893481 |
| Anti-mouse Ly-6G, FITC conjugated, clone: 1A8 | BD PharMingen | Cat# 551460; RRID:AB_394207 |
| Anti-mouse CD16/CD32 (Fc block), clone: 2.4G2 | BD PharMingen | Cat # 553142; RRID:AB_394657 |
| Anti-mouse CD3ε, PerCP-Cy5.5 conjugated, clone: 145-2C11 | BD PharMingen | Cat# 551163; RRID:AB_394082 |
| Anti-mouse CD8α, FITC conjugated, clone: 53-6.7 | BD PharMingen | Cat# 553031; RRID:AB_394569 |
| Anti-mouse CD4, FITC conjugated, clone: RM4-5 | BD PharMingen | Cat# 553047; RRID:AB_394583 |
| Anti-mouse CD11c, PE conjugated, clone: HL3 | BD PharMingen | Cat# 553802; RRID:AB_395061 |
| Anti-mouse CD103, BV421 conjugated, clone: M290 | BD PharMingen | Cat # 562771; RRID:AB_2737783 |
| Anti-mouse F4/80, FITC conjugated, clone: BM8 | BioLegend | Cat # 123108; RRID:AB_893502 |
| Anti-mouse B220, FITC conjugated, clone: RA3-6B2 | BioLegend | Cat # 103206; RRID:AB_312991 |
| Anti-mouse I-A/I-E (MHCII) Antibody, PE-Cy7 conjugated, clone: M5/114.15.2 | BioLegend | Cat# 107628; RRID:AB_2069377 |
| Anti-mouse CD8α, APC conjugated, clone: 53-6.7 | BD PharMingen | Cat# 553035; RRID:AB_398527 |
| Rabbit anti-mouse CD8α, clone: D4W2Z | Cell Signaling Technologies | Cat# 98941; RRID:AB_2756376 |
| Anti-mouse CXCL5 | LsBio | Cat # LS-C104413; RRID AB_10624103 |
| Rat anti-mouse Ly6G | Abcam | Cat# ab25377; RRID:AB_470492 |
| Rat anti-mouse IDO1, clone: mIDO-48 | Santa Cruz Biotechnology | Cat# sc-63978; RRID:AB_8131071 |
| Rabbit anti-TDO2 | Proteintech | Cat# 15880-1-AP; RRID:AB_2827610 |
| Rabbit anti-mouse β-Catenin, clone: D10A8 | Cell Signaling Technologies | Cat# 8480; RRID:AB_11127855 |
| Anti-mouse CD45, PerCp-Cy5.5 conjugated, clone:30-F11 | BD PharMingen | Cat# 550994; RRID:AB_394003 |
| Anti-mouse CD3ε, FITC conjugated, clone: 145-2C11 | BD PharMingen | Cat# 553061; RRID:AB_394594 |
| Anti-mouse CD8α, BV510 conjugated, clone: 53-6.7 | BD PharMingen | Cat# 563068; RRID:AB_2687548 |
| OMP-18R5 | OncoMed | N/A |
| OMP-54F28 | OncoMed | N/A |
| Anti-PD-1, clone: RMP-14 | BioXCell | Cat# BE0146; RRID:AB_10940953 |
| Anti-CD8 | Duke Cell Culture Facility | N/A |
| Rat IgG2a isotype control, clone: 2A3 | BioXCell | Cat# BE0089; RRID:AB_1107769 |
| **Bacterial and virus strains** | | |
| Ad5CMVCre | Univ. of Iowa | Cat # Ad5CMVCre |
| **Biological Samples** | | |
| Human melanoma tissue specimens | Duke University Hospital | N/A |
| Pre and Post ETC-159 treated human tumor specimens | A*STAR | N/A |
| **Chemicals, peptides, and recombinant proteins** | | |
| Collagenase IV | Sigma-Aldrich | Cat # C-5138 |
| Hyaluronidase | Sigma-Aldrich | Cat # H-6254 |
| DNase | Sigma-Aldrich | Cat # D-5025 |

(Continued on next page)
### Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| RPMI                | Sigma-Aldrich | Cat # R8758 |
| RBC Lysis Buffer    | Sigma-Aldrich | Cat # R7757 |
| Live/Dead Fixable Violet Dead Cell Stain Kit | ThermoFisher | Cat # L34955 |
| Live/Dead Fixable Aqua Dead Cell Stain Kit | ThermoFisher | Cat # L34966 |
| Recombinant Wnt5a   | R&D systems | Cat # 645-WN-010 |
| Aminolevulenic acid | Sigma   | Cat # A3785 |
| Hematoxylin         | VWR     | Cat # 95057-844 |
| Eosin               | VWR     | Cat # 95057-848 |
| Vina Green Chromogen Kit | BioCare Medical | Cat # BRR 807 AH |
| Warp Red Chromogen Kit | BioCare Medical | Cat # 901-WR806-081017 |
| NP40 lysis buffer   | Sigma-Aldrich | Cat # 492016 |
| RIPA Lysis and Extraction Buffer | ThermoFisher | Cat # 89901 |
| Protease Inhibitor Cocktail | Roche | Cat # 4693159001 |
| PhosSTOP            | Roche   | Cat # 4906845001 |
| TRP2180–188 peptide | ANASPEC | Cat # AS-61058 |
| MUT151–58 peptide   | BACHEM  | Cat # 4026648 |
| OVA257–264 peptide  | InvivoGen | Cat # vac-sin |
| C-59                | Selleckchem | Cat # S7037 |
| ETC-159             | A*STAR  | N/A |
| Collagenase IV      | Sigma-Aldrich | Cat # C-5138 |
| Recombinant Mouse IL-4 | BioAbChem | Cat # 42-IL4 |
| Recombinant Mouse GM-CSF | R&D System | Cat # 415-ML-010 |
| 4-hydroxytamoxifen  | Sigma-Aldrich | Cat # H6278-10MG |
| Polyethylene glycol(PEG)-8000 | Sigma-Aldrich | Cat # 89510-250G-F |

#### Critical commercial assays

- **Mouse Kynurenine ELISA kit**: MyBioSource [Cat # MBS043489](https://www.mybiosource.com)
- **Mouse IFNγ ELISPOT Plus**: Mabtech [Cat # 3321-4APT-2](https://www.mabtech.com)
- **Mouse FoxP3 Buffer Set**: BD Biosciences [Cat # 560409](https://www.bdbiosciences.com)
- **Miltenyi CD11c magnetic selection columns**: Miltenyi Biotec [Cat # 130-108-338](https://www.miltenyibiotec.com)
- **Naive CD4+ T cells selection kit**: Stem Cell Technologies [Cat # 19765](https://www.stemcelltech.com)
- **RNAeasy Plus Micro kit**: QIAGEN [Cat # 74034](https://www.qiagen.com)
- **SuperScript IV FirstStrand Synthesis System**: Invitrogen [Cat # 11756050](https://www.invitrogen.com)
- **PowerUp Master Mix**: Applied Biosciences [Cat # A25742](https://www.appliedbiosystems.com)

#### Deposited data

- **RNA-seq data derived from the murine BRAFV600Epten/-/- melanoma anti-PD-1 resistance study**: Hanks Lab; Theivanthiran et al., 2020; Zhao et al., 2018a [GEO: GSE165745](https://www.ncbi.nlm.nih.gov/geo)
- **RNA-seq dataset derived from metastatic melanoma patients prior to anti-PD-1 treatment**: Hugo et al., 2016 [GEO: GSE78220](https://www.ncbi.nlm.nih.gov/geo)
- **Wnt pathway-focused Nanostring dataset derived from metastatic melanoma patients prior to anti-PD-1 treatment**: This paper [GEO: GSE167039](https://www.ncbi.nlm.nih.gov/geo)
- **Nanostring data from ETC-159 treated patients**: This paper [GEO: GSE167039](https://www.ncbi.nlm.nih.gov/geo)

#### Experimental models: Cell lines

- **BrafV600Epten/-/- (BPD6, male) cell line**: Hanks Lab [N/A](https://www.hankslab.org)
- **p53foxofoxoKrasG12D cell line**: Hanks Lab [N/A](https://www.hankslab.org)
- **Lewis Lung Carcinoma**: ATCC [ATCC Cat# CRL-1642; RRID: CVCL_4358](https://www.atcc.org)
- **DC2.4**: Kenneth Rock, University of Massachusetts Medical School [Shen et al., 1997; RRID: CVCL_409](https://www.umassmedical.edu)

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| HEK293-LEF1/TCF-luciferase cell line | N/A | Ring et al., 2011 |
| B6.CgBRAF<sup>tm1Mmcm</sup>PTEN<sup>tm1Hwu</sup>Tg(TyrCre/ERT2)13Bos/BosJ (BRAF<sup>V600E</sup> PTEN<sup>+/−</sup>, H-2<sup>b</sup>) transgenic mice | Jackson Labs | IMSR Cat# JAX:013590; RRID:IMSR_JAX:013590 |
| C57BL/6 wild type | Jackson Labs | IMSR Cat# JAX:000664; RRID:IMSR_JAX:000664 |
| BALB/c (H-2<sup>b</sup>) wild type | Jackson Labs | IMSR Cat# JAX:000651; RRID:IMSR_JAX:000651 |
| B6.Cg-Foxp3<sup>tm2Tch</sup>/J (FoxP-EGFP, H-2<sup>b</sup>) | Jackson Labs | IMSR Cat# JAX:006772; RRID:IMSR_JAX:006772 |
| Oligonucleotides | | |
| Tcf7-forward: AGT TCT TCT CAC TCT AGG AAC A | IDT | N/A |
| Tcf7-reverse: AAT CCA GAG AGA TCG GGG GTC | IDT | N/A |
| Ccnd1-forward: GCG TAC CCT GAC ACC AAT CTC | IDT | N/A |
| Ccnd1-reverse: CTC CTC TCT GCA CTT CTC C | IDT | N/A |
| Gapdh-forward: GTG TAC ATG TTC CAG TAT GAC TCC | IDT | N/A |
| Gapdh-reverse: AGT GAG TTG TCA TAT TTC TCG TGG T | IDT | N/A |
| Cxcl2-forward: GAG CTT GAG TGT GAC GCC CCC AGG | IDT | N/A |
| Cxcl2-reverse: GTT AGC CTT GAC TGT CAG TAT TAC | IDT | N/A |
| Cxcl5-forward: GCA TTT CTC TTG CTG TAC AGC CTG | IDT | N/A |
| Cxcl5-reverse: CCT CCT TCT GTT TTC TTA GGA TAC C | IDT | N/A |
| Actb-forward: GTC TAC ATG TTC CAG TAT GAC TCC | IDT | N/A |
| Actb-reverse: AGT GAG TTG TCA TAT TTC TCG TGG T | IDT | N/A |
| Ccl4-forward: GGC CTC TCT CTC TCT TGTC | IDT | N/A |
| Ccl4-reverse: GTC TGC CTC TTT TGG TCA GG | IDT | N/A |
| Software and algorithms | | |
| nSolver | Nanostring | RRID: SCR_003420 |
| Amira 3D Visualization and Analysis Software Suite | FEI, Thermo Fisher Scientific | RRID: SCR_007353 |
| ImageJ | ImageJ | RRID: SCR_003070 |
| FlowJo Version 10 | Tristar | RRID: SCR_008520 |
| Prism | GraphPad | RRID: SCR_002798 |
| ImmunoCapture and ImmunSpot | ImmunoSpot | RRID: SCR_011082 |
| R | R Project for Statistical Computing | RRID:SCR_001905 |
| Heatmap.2 | R Function | https://cran.r-project.org/web/packages/gplots/index.html |
| Other | | |
| XRAD225 Cx | Precision X-Ray, Inc | N/A |
| gentleMACS Tissue Dissociator | Miltenyi Biotec | Cat # 130-093-235 |
| FACSCanto II | Becton Dickinson | N/A |
| LSR II | Becton Dickinson | N/A |
| gentleMACS C-tubes | Miltenyi Biotec | Cat # 130-093-237 |
| gentleMACS M-tubes | Miltenyi Biotec | Cat # 130-093-236 |
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact Brent A. Hanks, M.D., Ph.D. (brent.hanks@duke.edu).

Materials availability
This study did not generate new unique reagents.

Data and code availability
RNA-seq data derived from the BP melanoma anti-PD-1 resistance study is available on the Sequence Read Archive (SRA) database, accession number SAMN09878780 (Theivanthiran et al., 2020; Zhao et al., 2018a). RNA-seq data derived from the Hugo et al. human melanoma anti-PD-1 resistance study was originally deposited onto the GEO Database under accession number GEO: GSE78220 (Hugo et al., 2016). Nanostring data from metastatic melanoma patient samples prior to anti-PD-1 and from patients treated with ETC-159 were deposited to the GEO Database under the accession numbers GEO: GSE165745 and GSE167039, respectively.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Autochthonous and syngeneic mouse studies
Mouse experiments were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Duke University Medical Center. B6.CgBrafV600E/PTEN−/−Tg[TyrCre/ERT2]13Bos/BosJ (BRAFV600E/PTEN−/−, BP, H-2b) transgenic mice (Jackson Labs, IMSR Cat# JAX:013590, RRID:IMSR_JAX:013590) were sub-dermally injected with 4-HT (38.75 mg/mouse; Sigma-Aldrich, H6278-10MG) to induce primary melanoma development at the base of the tail. BP melanoma and Lewis lung carcinoma cell lines were implanted by subcutaneous injection at the base of the tail of syngeneic C57BL/6J mice (Jackson Labs, IMSR Cat# JAX_000664, RRID:IMSR_JAX:000664). Tumor bearing mice were randomized into treatment groups when tumors reached approximately 65-100 mm3 (0.065-0.1 cm3). Tumor volumes were calculated according to the following formulas:

\[
\text{cm}^3 = \frac{1}{2} (\text{length, cm}) \times (\text{width, cm})^2
\]

\[
\text{mm}^3 = \frac{1}{2} (\text{length, mm}) \times (\text{width, mm})^2
\]

All experimental groups included randomly chosen littermates of both sexes, ages 6–8 weeks, and of the same strain. Melanoma and LLC growth was monitored by orthogonal caliper measurements every 3-4 days. Normalized tumor volumes were used to display data related to the autochthonous melanoma model to account for variations in primary tumor size. KrasG12Dp53flox/flox (KP) mice (a gift from Anton Berns, the Netherlands Cancer Institute) were anesthetized with isoflurane and administered Ad5CMVCre (University of Iowa) adenovirus vector intra-nasally to induce lung adenocarcinoma development (DuPage et al., 2009). Mice ages 6-8 weeks and littermates of both sexes were randomly assigned to treatment cohorts 10 weeks after virus administration. These mice were monitored by micro-CT imaging as described below. Mice were treated with rat IgG2a isotype control (BioXCell, BE0089) or anti-PD-1 mAb at 200 μg/dose (BioXCell, BE0089, BE0146) every 3 days via intraperitoneal (i.p.) injection. OMP-18R5 and OMP-54F28 (OncoMed) were given weekly via i.p. injection at 400 μg/dose. ETC-159 (A*STAR) in polyethylene glycol (PEG, Sigma-Aldrich, 89510-250G-F) vehicle or PEG alone was given orally at 200 μg/dose every 3 days. CD8 depletion was conducted as previously described (Zhao et al., 2018a). B6.Cg-Foxp3tm2Tch/J (FoxP-EGFP, H-2b) transgenic mice and BALB/c mice (H-2d) were purchased from Jackson Labs (IMSR Cat# JAX_000651, RRID:IMSR_JAX:000651, IMSR Cat# JAX:006772, RRID:IMSR_JAX:006772) for performing Treg differentiation assays. All mice, regardless of experiment and strain were housed in an isolated animal facility requiring personal protective clothing for entry and monitored by Duke veterinary staff. Mice are housed in micro-isolator caging, up to 5 mice per cage, on corn cob bedding and changed every two weeks. Temperature, humidity and pressures of the mouse facility are controlled by a pneumatic control system with digital backup alarms. Exhaust systems and air supply is HEPA filtered. Species-specific heat and humidity are maintained within the parameters outlined in The Guide for the Care and Use of Laboratory Animals. All mice were euthanized by a carbon dioxide (CO2) euthanasia chamber followed by cervical dislocation.

Cell lines
BrafV600E/PTEN−/− (BPD6, male) cell lines were generated and cultured in DMEM with 10% FBS at 37°C as previously described (Holtzhausen et al., 2015). KrasG12Dp53flox/flox cell lines were generated through enzymatic and mechanical digestion of autochthonous mouse lungs during week 16 post-virus administration by visual tumor dissection and serial culturing in DMEM with 10% FBS to remove fibroblasts. Cell lines were confirmed by rt-PCR and western blot. Lewis Lung Carcinoma cells were purchased from the ATCC (ATCC, CRL-1642) and cultured according to the supplier’s specifications. Bone marrow-derived dendritic cells (BMDCs)
were harvested and differentiated using IL-4 (BioAbChem, 42-IL4 C) and GM-CSF (BioAbChem, 42-GMCSF) as previously described and purified using CD11c microbeads (Miltenyi Biotec, 130-108-338) according to the manufacturer’s protocol (Inaba et al., 1992). The HEK293-LEF1/TCF-luciferase cell line was cultured in DMEM with 10% FBS as previously described in the presence or absence of OMP-18R5 or OMP-54F28 (Ring et al., 2011). The murine dendritic cell line, DC2.4 (a generous gift from Dr. Kenneth Rock, University of Massachusetts Medical School) was cultured and generated as previously described (Shen et al., 1997).

**Human studies**

The archival melanoma tissue specimen study associated with this work was approved by the Duke University Medical Center institutional review board (IRB) (ClinicalTrials.gov: NCT02694865). Only treatment naive patients that underwent anti-PD-1 monotherapy with either pembrolizumab (200 mg IV every 3 weeks) or nivolumab (240 mg IV every 2 weeks) were included. All patients provided written consent and archival specimens were selected from available tissues. Patient demographics are provided in Table S2. Patient demographics associated with the tissue specimen studies derived from the Phase I ETC-159 clinical trial (ClinicalTrials.gov: NCT02521844) are described in Table S3.

**METHOD DETAILS**

**Flow cytometry**

Tumor tissues were resected and processed using the following tissue digestion mixture: collagenase IV (1 g/100mL HBSS, 10x stock, Sigma-Aldrich, C-5138), hyaluronidase (100 mg/100mL HBSS, 10x stock solution, Sigma-Aldrich, H-6254), DNasel (20,000 U/100mL HBSS, 10x stock solution, Sigma-Aldrich, D-5025) in serum free RPMI (Sigma-Aldrich, R8758) followed by mechanical dissociation with gentleMACS Tissue Dissociator (Miltenyi Biotec, 130-093-235) using gentleMACS-tubes (Miltenyi Biotec, 130-093-237) and incubation at 37°C with agitation at 250 rpm for 30 minutes. Single Cell suspension was lysed with red blood cell (RBC) lysis buffer (Sigma-Aldrich, R7757) according to the manufacturer’s protocol. One million cells were stained with 1 μg per million cells of each fluorochrome conjugated antibodies or commercially available dyes according to the standard protocols and analyzed using a FACScanto II or LSRII flow cytometer (Becton Dickinson). The following antibodies were used: Anti-mouse CD45, PerCp-Cy5.5 conjugated, clone:30-F11 (BD PharMingen, 550994). Anti-mouse CD3e, FITC conjugated, clone: 145-2C11 (BD PharMingen, 553061). Anti-mouse CD8a, BV510 conjugated, clone: 53-6.7 (BD PharMingen, 563068). Anti-mouse CD4, APC conjugated, clone: RM4-5 (BD PharMingen, 553051). Anti-mouse Foxp3, PE conjugated, clone: MF23 (BD PharMingen, 560408). Anti-mouse CD11b, PE conjugated, clone: M1/70 (BD PharMingen, 557397). Anti-mouse F4/80, APC conjugated, clone: BM8 (Biolegend, 132116). Anti-mouse Ly-6G, FITC conjugated, clone: 1A8 (BD PharMingen, 551460). Anti-mouse CD16/CD32 (Fc block), clone: 2.4G2 (BD PharMingen, 553142). Anti-mouse CD45, APC-Cy7 conjugated, clone: 30-F11 (BD PharMingen, 557859). Anti-mouse CD3e, PerCP-Cy5.5 conjugated, clone: 145-2C11 (BD PharMingen, 551163). Anti-mouse CD8a, FITC conjugated, clone: 53-6.7 (BD PharMingen, 553031). Anti-mouse CD4, FITC conjugated, clone: RM4-5 (BD PharMingen, 553047). Anti-mouse CD11c, PE conjugated, clone: HL3 (BD PharMingen, 553802). Anti-mouse CD103, BV421 conjugated, clone: M290 (BD PharMingen, 562771). Anti-mouse F4/80, FITC conjugated, clone: BM8 (Biolegend, 123108). Anti-mouse B220, FITC conjugated, clone: RA3-6B2 (Biolegend, 103206). Anti-mouse I-A/I-E (MHCII) Antibody, PE-Cy7 conjugated, clone: M5/14.15.2 (Biolegend, 107628). Anti-mouse CD8a, APC conjugated, clone: 53-6.7 (BD PharMingen, 553035). Non-viable cells were excluded from further flow analysis using a Live/Dead Fixable Violet Dead Cell Stain Kit (ThermoFisher, L34955) or Aqua Dead Cell Stain Kit (ThermoFisher, L34966). FoxP3 staining was conducted per the manufacturers protocol using the mouse FoxP3 Buffer Set (BD Biosciences, 560409). Data were analyzed using FlowJo version 10. All gating strategies are described in Figure S6D.

**Protoporphyrin IX flow cytometry**

DC2.4 or BrafV600E/Pten−/− melanoma cells were cultured for 48 hours with OMP-18R5 and/or recombinant Wnt5a (R&D systems, 645-WN-010), terminally incubated for 4 hours with aminolevulenic acid (Sigma-Aldrich, A3785) followed by flow cytometry (BD Fortessa Il) as previously described (Zhao et al., 2018).

**Immunohistochemistry**

Tissues were paraffin embedded and processed following standard protocols and imaged with a Zeiss CLSM 700 confocal microscope. For Lung and Tumor H&E, hematoxylin (VWR, 95057-844) was stained followed by Eosin (VWR, 95057-844). The following antibodies were used for Immunohistochemistry: Rabbit anti-mouse CD8 (D4W2Z) 1:400 (Cell Signaling, 98941), anti-mouse CXCL5 1:200 (LSBio, LS-C104413), rat anti-mouse Ly6g 1:100 (Abcam, ab25377), rabbit anti-mouse β-catenin 1:100 (Cell signaling, 8480). Antigen retrieval was performed by incubation in BioCare’s Nexgen Decloaker chamber for 20 minutes at 90°C. Anti-rabbit polymers were used as secondary antibodies and Vina Green Chromogen Kit (Biocare Medical, BRR 807 AH) was used as substrate for CDB. For CXCL5, LY6G, and β-catenin, anti-rat or rodent polymers were used as secondary antibodies, and Warp Red Chromagen Kit (Biocare Medical, 901-WR806-081017) was used as substrate. ImageJ software was used for quantification.
Western blot
Tumor tissue or cells were homogenized in 1% NP40 lysis buffer (Sigma-Aldrich, 492016) or RIPA Lysis and Extraction Buffer (ThermoFisher, 89901) supplemented with complete protease inhibitor and phosphatase inhibitor (Roche, 4693159001 and 4906845001). Protein samples were separated by SDS-PAGE and transferred onto PVDF/Nitrocellulose membranes (Bio-Rad). Monoclonal primary antibodies and appropriate HRP-conjugated secondary antibodies were used for blotting. The proteins were visualized by ECL-Plus (GE Healthcare) using the Syngene G Box system (Syngene) or ImageQuant LAS 500 (GE HealthcareLife Sciences). The following antibodies were used: IDO1 (mIDO-48, Santa Cruz Biotechnology, sc-53978) 1:500 overnight at 4°C, TRP2180-188 peptide (1 mg/mL, SVYDFFVWL; ANASPEC, AS-61058) for melanoma experiments, MUT151-58 peptide (1 mg/mL, FEQNTAQP; BACHEM, 4026648) for Lewis Lung Carcinoma experiments, ConA-positive control, or the irrelevant negative control OVA257–264 peptide (1 mg/mL, SIINFEKL, InvivoGen). To perform the tumor cell lysate ELISPOT assay for the KP autochthonous lung cancer experiment, we modified a previously described method. Briefly, 10^7 monolayer cultures were trypsinized, washed with cold PBS thrice, and resuspended in 1 mL of PBS. The cells were frozen and thawed four times, and the lysate was centrifuged at 300 g for 10 min. The supernatant concentration was determined using a Pierce BCA Protein Assay Kit (Thermo Fisher, 23227). Tumor lysate was added to splenocytes at a final concentration of 120 μg/mL and incubated for 24 hours. Imaging was conducted using a CTL ImmunoSpot S5 core (ImmunoSpot) and quantified using ImmunoCapture and ImmunoSpot software (ImmunoSpot).

ELISPOT assays
Mouse IFNγ ELISPOTPLUS (MABTECH) was performed according to the manufacturer’s guidelines. In brief, single-cell suspensions of splenocytes, generated by mechanical dissociation followed by RBC lysis using ammonium chloride, were plated at 2.5x10^5–1x10^6 cells/well on an ELISPOT plate (MABTECH, 3321-4APT-2) and incubated for 24 hours at 37°C with the following peptides: TRP2180-188 peptide (1 mg/mL, SVYDFFVWL; ANASPEC, AS-61058) for melanoma experiments, MUT151-58 peptide (1 mg/mL, FEQNTAQP; BACHEM, 4026648) for Lewis Lung Carcinoma experiments, ConA-positive control, or the irrelevant negative control OVA257–264 peptide (1 mg/mL, SIINFEKL, InvivoGen). To perform the tumor cell lysate ELISPOT assay for the KP autochthonous lung cancer experiment, we modified a previously described method. Briefly, 10^7 monolayer cultures were trypsinized, washed with cold PBS thrice, and resuspended in 1 mL of PBS. The cells were frozen and thawed four times, and the lysate was centrifuged at 300 g for 10 min. The supernatant concentration was determined using a Pierce BCA Protein Assay Kit (Thermo Fisher, 23227). Tumor lysate was added to splenocytes at a final concentration of 120 μg/mL and incubated for 24 hours. Imaging was conducted using a CTL ImmunoSpot S5 core (ImmunoSpot) and quantified using ImmunoCapture and ImmunoSpot software (ImmunoSpot).

Treg assay
BMDCs from BALB/c mice were treated with either OMP-18R5 or OMP-54F28 for 2 hours followed by treatment with Wnt5a (200 ng/mL) (R&D systems) for 48 hours. For BrafV600E/Pten−/− melanoma cell line derived conditioned-media, melanoma cells were treated with C59 at 100 nmol/L (Selleckchem, S7037) or DMSO control for 48 hours, followed by stimulation of BMDCs as previously described (Holtzhausen et al., 2015) for 48 hours. BMDCs were washed and CD11c selection was performed using the Miltenyi CD11c magnetic selection columns (Miltenyi, 130-108-338), per the manufacturer’s instructions. cDNA was generated using the SuperScript IV FirstStrand Synthesis System (Invitrogen, 11756050) and qRT-PCR was performed on an AB 7500 Real-Time PCR Instrument (Life Technologies) using the PowerUp Master Mix (Applied Biosciences, A25742). Ct values were normalized to GAPDH using the Ct method.

Tumor RNA isolation and qRT-PCR
Flash frozen tumors were processed with M-tubes (Miltenyi, 130-093-236) in RLT PLUS (Qiagen, 1053393) with β-mercaptoethanol (VWR, VWRV0482-250ML). RNA was isolated using the RNAeasy Plus Micro kit (Qiagen, 74034) per the manufacturer’s instructions. cDNA was generated using the SuperScript IV FirstStrand Synthesis System (Invitrogen, 11756050) and qRT-PCR was performed on an AB 7500 Real-Time PCR Instrument (Life Technologies) using the PowerUp Master Mix (Applied Biosciences, A25742). Ct values were normalized to GAPDH using the ΔΔCt method.

Lung imaging and analysis
KP mice were imaged for tumor development by serial micro-CT at 2-week intervals beginning 10 weeks post infection as previously described (Perez et al., 2013; Torok et al., 2019). Briefly, the XRAD225 Cx (Precision X-Ray, Inc.) small-animal image-guided irradiator was used for serial micro-CT. Mice were placed in a prone position and anesthetized with isofluorane via nose cone delivery system. Mice were breathing freely during image acquisition, thus tumor volumes represented an internal target volume that integrated changes over the respiratory cycle. For CT acquisition to image the lungs 40 kVP, 2.5 mA X-rays were used with a 2-mm Al filter,
and images were analyzed using the Amira 3D Visualization and Analysis Software Suite (FEI, ThermoFisher Scientific) to calculate tumor volumes as further described elsewhere (Torok et al., 2019). Each individual lesion was contoured to calculate the tumor volume. In most mice, multiple target lesions were identified. Target lesion volume was subsequently measured every 2 weeks and normalized to the volume at baseline to calculate fold changes in tumor volume. Target lesions obscured by other anatomic changes were excluded.

**Kynurenine LC-MS/MS**

LC-MS/MS was performed by the Duke Pharmacokinetics/Pharmacodynamics core facility. Kyn-KynA-Trp working solutions were as followed: Kyn stock solution (SS1): 5 μg/mL, Trp stock solution (SS2): 1 mg/mL, KynA stock solution (SSA): 20 μg/mL. The following calibration curves were: Kyn: 2.6 – 100 ng/mL, KynA: 0.5-20 ng/mL, Trp: 0.5-20 μg/mL. Drug extraction was performed on tumor homogenate consisting of 1x tissue and 2x H2O. 60 μL IS (100 ng/mL Kyn-d4+ 1 ug/mL Trp-d5+20 ng/mL KynA-d5) in mphA was added to 20 μL of homogenate. 200 μL of CH3Cl was then added to mixture. Samples were homogenized/extracted on “Fast Prep” at Speed 4.0 for 20 s and centrifuged for 5 minutes at 16,000 g. For Trp, 2 μL of supernatant was transferred into an injection vial, 40 μL of mphA was added, and the sample was vortexed, centrifuged, and 5 μl was injected. For Kyn/KynA: the leftover supernatant was transferred into another injection vial. Samples were then run on 4000Q LC/MS/MS system at 4°C. Example chromatographs are demonstrated in Figure S5F.

**RNaseq dataset re-analysis**

RNA-seq data from Hugo et al. (GEO: GSE78220) (Hugo et al., 2016) was processed using the TrimGalore toolkit which employs Cutadapt to trim low-quality bases and Illumina sequencing adapters from the 3’ end of the reads. Only reads that were 20nt or longer after trimming were kept for further analysis. Reads were mapped to the GRCh37v73 version of the human genome and transcriptome using the STAR RNA-seq alignment tool. Reads were excluded for subsequent analysis if they mapped to a single genomic location. Gene counts were compiled using the HTSeq tool. Only genes that had at least 10 reads in any given library were used in subsequent analysis. Normalization and differential expression were carried out using the DESeq2 Bioconductor package with the R statistical programming environment. We included batch and sex as cofactors in the differential expression model. The false discovery rate was calculated to control for multiple hypothesis testing. Gene set enrichment analysis was performed to identify differentially regulated pathways and gene ontology terms for each of the comparisons performed. Wnt pathway focused re-analysis was performed on RNaseq from autochthonous mouse melanomas treated with IgG or anti-PD-1 as previously described (50-bp single-read sequencing; Anti–PD-1 resistance Study RNA-seq, accession number: SAMN09878780) (Zhao et al., 2018a). Heatmaps were generated using the heatmap.2 function in R.

**NanoString**

Archival melanoma specimens of patients treated with anti-PD-1 (either pembrolizumab or nivolumab) monotherapy were obtained via an ongoing clinical trial (ClinicalTrials.gov: NCT02694965). After verification by a board-certified pathologist, microdissection was performed if deemed necessary. Two-to-three 10 μM FFPE scrolls were collected from FFPE sectioning of biopsies taken prior to treatment, and RNA extraction was performed using RSC FFPE RNA extraction kit (Promega) according to manufacturer’s instructions. Agilent 2100 Bioanalyzer (Agilent) and NanoDrop (ThermoFisher) were used to determine the purity and concentration of the RNA. A DV300 Agilent Bioanalyzer smear analysis was used to determine the percentage of fragments greater than 300 nucleotides to determine the starting amount of RNA. Samples were analyzed on a NanoString nCounter Max system using the nCounter Vantage 3D Human Wnt Pathways Panel (N2_WNT_Pth_v1.0), which is comprised of Wnt ligands, receptors, regulators, gene targets, and other pathway components with the addition of a custom gene panel composed of cytolytic T cell markers and IDO1. Gene expression codesets (designed and produced by NanoString Technologies, Seattle, WA), hybridization buffer and total RNA were hybridized in a thermocycler for 16 hours at 67°C prior to being processed in the nCounter Max Prep Station following the NanoString manual, MAN-10056-02 for gene expression assays. Data collection using the nCounter Digital Analyzer was performed by following the NanoString manual, MAN-C0035-07 using the maximum field of view setting. Analysis was performed using the nSolver software (NanoString). Tumor specimen samples from the multi-site, international, phase I advanced solid tumor ETC-159 clinical trial (ClinicalTrials.gov: NCT02521844, A’STAR Research Entities, Singapore), were analyzed using the PanCancer IO panel (NS_CANCERIMMUNE_) which contains immune genes including cytokines, chemokines, myeloid cells, T-, B-, and NK-cells, and genes associated with antigen processing.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

GraphPad Prism 7 Windows version was used for all statistical analyses. Unpaired two-sided Student’s t tests were used to compare mean differences between control and treatment groups. Univariate ANOVA followed by Tukey post hoc test was performed to analyze data containing three or more groups. Kaplan-Meier estimates were utilized for survival analyses. The Benjamini-Yekutieli method of correction was utilized to control the false-discovery rate (FDR) associated with the Nanostring transcriptional data analysis. The significance threshold for all statistical calculations was based on a P value of 0.05, and all tests were two-sided. All quantitative data is presented as a mean and associated SEM. Number of replicates and individual tests used can be found in
the figure legends. Mice were allocated to treatment groups to maintain similar average initial tumor sizes in each group. No data was excluded in described studies.

ADDITIONAL RESOURCES

Archival melanoma specimens of patients treated with anti-PD-1 (either pembrolizumab or nivolumab) monotherapy were obtained via an ongoing clinical trial (ClinicalTrials.gov: NCT02694965). Patient samples were also obtained from the completed multi-site, international, phase I advanced solid tumor ETC-159 clinical trial (ClinicalTrials.gov: NCT02521844, A*STAR Research Entities, Singapore).
Supplemental information

Pharmacological Wnt ligand inhibition overcomes key tumor-mediated resistance pathways to anti-PD-1 immunotherapy

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Figure S1.

A. Bar graph showing relative light units (RLU) for different conditions.

B. Graph showing β-catenin Allred Score with different treatments.

C. Bar graph showing percentage of CD4+FOXP3+ Tregs.

D. Images showing IgG Isotype Ctrl, α-PD-1 mAb, and combinations with OMP-18R5 and OMP-54F28.

E. Images showing CD8-Blue staining with α-PD-1 mAb treatments.
**Figure S1. Supportive Studies for In vitro and In vivo Wnt Inhibitor Experiments. Related to Figure 2.**  
(A) Luciferase assay of the TCF-LEF-luc 293T cell line treated with OMP-18R5 at increasing concentrations. Each condition performed in triplicate. Data is representative of two independent experiments. Associated with Figure 2A. (B) Immunohistochemistry staining for β-catenin of syngeneic BRAF^{V600E}PTEN^-/- melanoma tumors following treatment with IgG ctrl, OMP-18R5, or OMP-54F28. *Left*, staining intensity determined based on both area and intensity (derived from the Allred scoring system in breast cancer). *Right*, representative images from 10 random fields for 3 tumors from each of 3 treatment conditions. 20x. scale bar, 50 μm. Associated with Figure 2E. (C) DCs were treated with Wnt5a ± OMP-54F28 followed by co-culture with naïve CD4^+ T cells derived from FoxP3-GFP reporter mice. CD4^+GFP^- Tregs were quantified by flow cytometry. Associated with Figure 2C,D. (D) Representative images of H&E analysis of lung metastasis following treatment with IgG ctrl, OMP-54F28/aPD1, and OMP-18R5/aPD1. *Red arrows*, metastatic foci. Ten random fields evaluated for each lung from each of 3 treatment conditions. 3 mice per group. 20x. scale bar, 18 μm. Associated with Figure 2F. (E) Representative images of tumor CD8 immunohistochemistry (blue stain). *Red arrows*, tumor-infiltrating CD8^+ T cells. 20x. scale bar, 50 μm. Associated with Figure 2G. All statistical analysis based on one-way ANOVA followed by Tukey post-hoc test. All data = mean +/- SEM. ***P<0.001, *P<0.05.
Figure S2.

A) Tumor Volume (cm³)

B) CD8⁺ T Cells

C) IgG Isotype Ctrl
OMP18R5
α-PD-1 mAb
α-PD-1 mAb + OMP18R5

D) OD (590 nm)

E) 4-HT
OMP18R5
α-PD-1
α-CD8

F) Normalized Lung Mets
Figure S2. Wnt Inhibition with OMP-18R5 Enhances Primary Tumor Control of the Autochthonous BRAF<sup>V600E</sup>PTEN<sup>-/-</sup> Melanoma Model in a Manner that Depends on the Host Immune System. Related to Figure 3. (A) Mean data for each treatment group derived from the autochthonous BRAF<sup>V600E</sup>PTEN<sup>-/-</sup> experiment described in Figure 3A. (B) Flow cytometry analysis of intra-tumoral CD8<sup>+</sup> T cells and Tregs following treatment of the autochthonous BRAF<sup>V600E</sup>PTEN<sup>-/-</sup> model with IgG ctrl, aPD1, OMP-18R5, or OMP-18R5/aPD1 (n=4-5/group). Data is representative of two independent experiments. Data associated with Figure 3C. (C) Images of IFN-γ ELISpot wells associated with Figure 3D. (D) BRAF<sup>V600EPTEN</sup><sup>-/-</sup> melanoma cell proliferation measured using a MTT assay following treatment with titrated levels of OMP-18R5 or OMP-54R28. Performed in triplicate. UT, untreated. ns, non-significant. (E) Schematic describing the treatment protocol associated with Figure 3E. 4-HT, 4-hydroxytamoxifen. Below, representative flow dot plot illustrating CD8<sup>+</sup> T cell ablation. (F) Measurement of lung metastases following treatment with IgG ctrl, OMP-18R5, or OMP-18R5/aPD1 in the presence or absence of CD8<sup>+</sup> T cells. Data normalized to IgG ctrl (n=3-4/group). Associated with Figure 3E. Student’s two-tailed t test was performed when comparing aPD1 monotherapy groups vs. combination therapy. All data = mean +/- SEM. **P<0.01, *P<0.05.
Figure S3.

(A) Tumor Volume (cm$^3$) over Days

(B) # Metastatic Foci Per Lung

(C) CD8$^+$ T cell/Treg Ratio

(D) # IFN-$\gamma$ Spots/1 x 10$^6$ Splenocytes

Legend:
- IgG Ctrl
- OMP54R28
- $\alpha$-PD-1 mAb
- OMP54R28/$\alpha$-PD-1 mAb

* Significant difference

IgG Isotype Ctrl
$\alpha$-PD-1 mAb
OMP54R28
$\alpha$-PD-1 mAb + OMP54R28
Figure S3. OMP-54FR28 Enhances the Efficacy of Anti-PD-1 Immunotherapy in the Autochthonous BRAF<sup>V600E</sup>PTEN<sup>-/-</sup> Melanoma Model. (A) Autochthonous BRAF<sup>V600E</sup>PTEN<sup>-/-</sup> mice were treated with either IgG isotype ctrl, aPD1, OMP-54R28, and OMP-54R28/aPD1 after the development of primary melanomas (n=6-7/group). (B) Quantification of lung metastasis in mice derived from (A) by H&E microscopy (n=3/group). (C) Flow cytometry analysis of intra-tumoral CD8<sup>+</sup> T cells and CD4<sup>+</sup>FoxP3<sup>+</sup> T cells in mice derived from (A). Data expressed as CD8<sup>+</sup> T cell:CD4<sup>+</sup>FoxP3<sup>+</sup> T cell ratios. (D) Quantification of TRP2-specific CD8<sup>+</sup> T cells derived from (A) based on IFN-γ ELISpot. Right, representative IFN-γ ELISpot wells. All statistical analysis based on one-way ANOVA followed by Tukey post-hoc test. All data = mean +/- SEM. **P<0.01, *P<0.05.
Figure S4.

A) Normalized Tumor Volume over Days Post-Implantation.

B) % CD3+CD8+ T Cells and CD8+ T cell/Treg Ratio.

C) # IFN-γ Spots/0.5 x 10^6 Splenocytes.

D) Normalized Tumor Volume.

E) BALF CD8+ T Cells.

F) % of Viable CD45+ Cells.

G) CD8-Light Blue staining.
Figure S4. OMP-18R5 Sensitizes the Lewis Lung Carcinoma Model and an Autochthonous Non-Small Cell Lung Cancer Model to Immunotherapy. Related to Figure 4. (A) Mice implanted with the LLC cell line were treated with either IgG isotype ctrl, aPD1, OMP-18R5, and OMP-18R5/aPD1 after the development of primary tumors (n=6/group). (B) Flow cytometry analysis of intra-tumoral CD8+ T cells and CD4+FoxP3+ T cells in mice derived from (A). Right, Data expressed as CD8+ T cell:CD4+FoxP3+ T cell ratios (n=3-4/group). (C) Quantification of TRP2-specific CD8+ T cells derived from (A) based on IFN-γ ELISpot (n=4). Right, representative IFN-γ ELISpot wells. (D) Autochthonous Kras^{G12D}p53^{flox/flox} mice were administered an inhaled adeno-cre viral vector resulting in primary lung tumor development. These mice were initiated on IgG isotype ctrl, OMP-18R5, aPD1, or OMP-18R5/aPD1 at week 10. Final tumor volumes at week 16 shown. (E) Representative micro-CT images of lung tumors for each treatment group. Yellow circles, primary lung tumor. (F) CD8+ T cell flow cytometry analysis of bronchoalveolar lavage fluid (BALF) harvested from mice in each treatment group described in (D) (n=4-6/group). (G) Representative images of CD8+ T cell IHC (light blue). Red arrows, tumor-infiltrating CD8+ T cells. Scale bars, 20 µm. All statistical analysis based on one-way ANOVA followed by Tukey post-hoc test. All data = mean +/- SEM. **** P<0.0001, **P<0.01, *P<0.05.
Figure S5.

#### A

![Image](https://via.placeholder.com/150)

**BRAF^{V600E/PTEN^{-}} Cell Line**

- OMP-18R5
- OMP-18R5

- Wnt5a
- TDO2
- IDO1
- β-actin

#### B

**DC2.4 Cell Line**

- OMP-18R5
- OMP-18R5

- Wnt5a
- IFNγ

- β-actin

#### C

**Tumor Cells**

- Wnt5a
- UT

**Protoporphyrin IX**

#### D

- IgG Ctrl
- α-PD-1 mAb + Epacadostat
- Anti-PD-1 mAb
- OMP18R5
- Epacadostat

#### E

**Tumor**

- ng/g of Kyn

**TDLN**

- ng/g of Kyn

#### F

**Kyn TUMOR #1**

- Kyn
- Kyn-dδ

**Kyn TDLN #1**

- Kyn
- Kyn-dδ

**Kyn NDLN #1**

- Kyn
- Kyn-dδ
Figure S5. Supportive Data for Kynurenine Inhibition Studies. Related to Figure 5.  

(A) BRAF<sup>V600E</sup>PTEN<sup>-/-</sup> melanoma cells were treated with or without Wnt5a in the presence or absence of OMP-18R5 followed by Western blot analysis for IDO1 and TDO2. Data representative of two independent experiments. 

(B) DCs were treated with Wnt5a in the presence or absence of OMP-18R5 followed by TDO2 Western blot analysis. 

(C) BRAF<sup>V600E</sup>PTEN<sup>-/-</sup> melanoma cells were treated with Wnt5a in the presence or absence of OMP-18R5 followed by terminal incubation in ALA and flow cytometric analysis for PpIX. 

(D) Representative IFN-γ ELISPOT images associated with Figure 5D. 

(E) Kynurenine ELISA of whole tumor and TDLN tissues following treatment with either OMP-18R5 or Epacadostat (n=4/group). Associated with Figure 5E. 

(F) Representative chromatograms from LC-MS/MS studies evaluating kynurenine and tryptophan levels in tumor, TLDN, and NDLN tissues resected from tumor-bearing mice. Kyn-d4 represents the internal standard. Associated with Figure 5E. All statistical analysis based on one-way ANOVA followed by Tukey post-hoc test. All data = mean +/- SEM. *P<0.05. **P<0.01
Figure S6.

A

B

C

D

Selection of live CD45 cells

CD45+/ Live

CD11b/ F4/80

CD45/ Ly6G

CD45/ MHCII

CD4+/ CD8

CD4+ FoxP3+ Tregs

CD45/ PMN-MDSCs

CD8+ T cells

CD45/ CD8

CD4/ CD3

CD4/ CD3

CD4+ Ly6G+ F4/80- cells/tumor tissue

IgG Ctrl
α-PD-1 mAb
α-PD-1 mAb/ETC-159

Log Fold vs. IgG

Tumor - Cxcl2

β-Catenin Targets

TCF7

CCND3

G1-Ly6G+ F4/80- cells/tumor tissue

IgG Ctrl
OMP18R5
α-PD-1 mAb
OMP18R5/α-PD-1 mAb

Log Fold Change

GRC Pre tx
GRC Post tx
Cholangiocarcinoma Pre tx
Cholangiocarcinoma Post tx

GRC Pre tx
GRC Post tx
Cholangiocarcinoma Pre tx
Cholangiocarcinoma Post tx
Figure S6. Wnt Inhibition Suppresses Tumor PMN-MDSC Recruitment. Related to Figures 6 and 7. (A) QrtPCR analysis of Cxcl2 expression in the autochthonous BRAF\textsuperscript{V600E}PTEN\textsuperscript{-/-} melanoma model after treatment with IgG control, aPD1 alone or with the addition of ETC-159 (n=6-8/group). Data representative of two experiments. Associated with Figure 6D. (B) Flow cytometry analysis of intra-tumoral CD11b\textsuperscript{+}Ly6G\textsuperscript{+}Ly6C\textsuperscript{lo}F4/80\textsuperscript{-} PMN-MDSCs normalized by tumor size. Tissue samples derived from experiments described in Figure 3A. (C) β-catenin target heatmaps (log-fold change) generated based on Nanostring analysis of tissue biopsies before and after ETC-159 treatment. CRC, colorectal cancer. Tx, treatment. Data associated with Figure 7. (D) Flow cytometry gating strategy for PMN-MDSCs, Tregs, CD103\textsuperscript{+} DCs, and CD8\textsuperscript{+} T cells used throughout this manuscript. Students two-tailed t test was performed to compare anti-PD-1 monotherapy groups vs. combination therapy. All data = mean +/- SEM. **P<0.01.
Figure S7.

A

% of CD103+ DCs

IgG Ctrl  OMP18R5  α-PD-1 mAb  OMP18R5/α-PD-1 mAb

% of CD103+ DCs

IgG Ctrl  ETC-159  α-PD1 mAb  ETC-159/α-PD-1 mAb

B

Tumor - Ccl4

Log Fold Change vs. IgG

IgG  Epacadostat  OMP18R5  anti-PD-1 mAb  Epacadostat/anti-PD-1 mAb

Log Fold Change vs. Pre-escape aPD1

PD1 post  anti-PD-1 mAb + dETC-159

C

ATF3

ns

Nonresponders  responders

CCL4

ns

Nonresponders  responders
Figure S7. Wnt Inhibition Does Not Significantly Alter Tumor CCL4 Expression or DC Recruitment. Related to Figures 3 and 5. **(A)** Intra-tumoral flow cytometric quantification of CD103⁺CD8⁺CD11c⁺ DC populations in autochthonous BRAFV600EPTEN⁻/⁻ melanoma mice treated with: Left, IgG isotype control or aPD1 with or without OMP-18R5 (n=4-5/group). Associated with Figure 3A. Right, IgG isotype control or aPD1 with or without delayed ETC-159. Associated with Figure 3F. **(B)** Left, qrtPCR analysis of Ccl4 expression levels in autochthonous BRAFV600EPTEN⁻/⁻ melanomas following treatment with IgG isotype ctrl, aPD1, OMP-18R5, Epacadostat, OMP-18R5/aPD1, or Epacadostat/aPD1 (n=3-5/group). Data associated with Figure 5C. Right, qrtPCR analysis of Ccl4 expression by autochthonous melanomas following aPD1 treatment and following the introduction of delayed ETC-159. Data associated with Figure 3F. **(C)** Expression of ATF3 (top) and CCL4 (bottom) from the Hugo et al (11) RNAseq analysis of patients with metastatic melanoma receiving aPD1 treatment annotated by response. ns, non-significant. All statistical analysis based on one-way ANOVA followed by Tukey post-hoc test in groups of 3 or more. Student’s two-tailed $t$ test was performed when comparing 2 groups. All data = mean +/- SEM.
**Table S1.** Related to Figure 1C. Log-fold Change and P-Value of Wnt-related Gene Expression in Anti-PD-1 Nonresponders versus Responders. Data derived from (Hugo et al., 2016).

| Gene    | Log-fold Change in Nonresponders | p-value     |
|---------|----------------------------------|-------------|
| WNT2    | 1.783996354                     | 0.012318688 |
| WNT5A   | 2.184619876                     | 0.00033232  |
| WNT5B   | 1.254066061                     | 0.019596701 |
| WNT7B   | 5.186058327                     | 1.66E-06    |
| WNT9A   | 1.747956784                     | 0.003339839 |
| WNT11   | 1.296378484                     | 0.045560098 |
| WNT16   | 1.964607655                     | 0.033351494 |
| FZD4    | 2.375837617                     | 0.017509166 |
| FZD8    | 3.932136645                     | 8.42E-05    |
| RSPO4   | 3.640103711                     | 0.000272528 |
| ROR2    | 2.778925952                     | 0.005453896 |
| CTNNA2  | 1.657617832                     | 0.040100106 |
| RNF43   | -3.192919043                    | 0.001408424 |
| Response    | Age at Biopsy | Gender | Location of Biopsy     | Treatment     |
|-------------|---------------|--------|------------------------|---------------|
| Responder   | 82            | F      | Skin metastasis        | Pembrolizumab |
| Responder   | 73            | M      | Lung metastasis        | Nivolumab     |
| Responder   | 77            | M      | Lymph Node             | Nivolumab     |
| Responder   | 76            | M      | Ileal metastasis       | Pembrolizumab |
| Responder   | 69            | F      | Adrenal Metastasis     | Pembrolizumab |
| Responder   | 84            | M      | Skin metastasis        | Pembrolizumab |
| Responder   | 70            | M      | Skin metastasis        | Nivolumab     |
| Responder   | 72            | M      | Lymph node             | Nivolumab     |
| Responder   | 82            | M      | Lymph node             | Nivolumab     |
| Responder   | 80            | M      | Skin metastasis        | Pembrolizumab |
| Responder   | 73            | M      | Lung metastasis        | Nivolumab     |
| Responder   | 75            | M      | Anal metastasis        | Pembrolizumab |
| Nonresponder| 55            | M      | Lymph Node             | Nivolumab     |
| Nonresponder| 49            | F      | Anal metastasis        | Pembrolizumab |
| Nonresponder| 57            | F      | Skin metastasis        | Nivolumab     |
| Nonresponder| 68            | F      | Lung metastasis        | Pembrolizumab |
| Nonresponder| 34            | M      | Lymph node             | Pembrolizumab |
| Nonresponder| 66            | F      | Skin metastasis        | Nivolumab     |
| Nonresponder| 77            | M      | Skin metastasis        | Pembrolizumab |
| Nonresponder| 70            | M      | Brain metastasis       | Pembrolizumab |
| Nonresponder| 73            | M      | Lymph metastasis       | Nivolumab     |
| Nonresponder| 66            | M      | Lymph node             | Nivolumab     |
| Nonresponder| 60            | M      | Skin metastasis        | Pembrolizumab |
| Nonresponder| 81            | M      | Parotid gland metastasis| Nivolumab   |

Table S2. Related to Figure 1D. Patient Demographics Associated with Melanoma Anti-PD-1 Response - Wnt Signaling Pathway Nanostring Study (NCT02694965).
### Patient Demographics Associated with Tissue Specimens Derived from the ETC-159 Phase I Clinical Trial (NCT02521844)

| Cancer Type            | Age | Gender |
|------------------------|-----|--------|
| Colon Cancer           | 70  | F      |
| Cholangiocarcinoma     | 62  | M      |

**Table S3. Related to Figure 7.**
### Table S4. Related to Figure 7.

Immune-related Gene Expression Changes Pre- and Post-ETC-159 Therapy. All data derived from Nanostring transcriptional analysis from tissue specimens harvested during the phase I ETC-159 clinical trial (NCT02521844).