**Melanoma-derived Wnt5a Promotes Local Dendritic-Cell Expression of IDO and Immunotolerance: Opportunities for Pharmacologic Enhancement of Immunotherapy**

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Abstract

The β-catenin signaling pathway has been demonstrated to promote the development of a tolerogenic dendritic cell (DC) population capable of driving regulatory T-cell (Treg) differentiation. Further studies have implicated tolerogenic DCs in promoting carcinogenesis in preclinical models. The molecular mechanisms underlying the establishment of immune tolerance by this DC population are poorly understood and the methods by which developing cancers can co-opt this pathway to subvert immune surveillance are currently unknown. This work demonstrates that melanoma-derived Wnt5a ligand upregulates the durable expression and activity of the indoleamine 2,3-dioxygenase-1 (IDO) enzyme by local DCs in a manner that depends upon the β-catenin signaling pathway. These data indicate that Wnt5a-conditioned DCs promote the differentiation of Tregs in an IDO-dependent manner and that this process serves to suppress melanoma immune surveillance. We further show that the genetic silencing of the PORCN membrane-bound O-acyl transferase, which is necessary for melanoma Wnt ligand secretion, enhances antitumor T-cell immunity and that the pharmacologic inhibition of this enzyme synergistically suppresses melanoma progression when combined with anti-CTLA-4 antibody therapy. Finally, our data suggest that β-catenin signaling activity, based on a target gene expression profile that includes IDO in human sentinel lymph node-derived DCs, is associated with melanoma disease burden and diminished progression-free survival. This work implicates the Wnt-β-catenin signaling pathway as a novel therapeutic target in the melanoma immune microenvironment and demonstrates the potential impact of manipulating DC function as a strategy for optimizing tumor immunotherapy.

Introduction
The generation of a tumor-specific immune response is critically dependent upon the antigen-presentation machinery of local dendritic cell (DC) populations residing in the tumor and tumor-draining lymph node (TDLN) tissues. By continuously sampling the tumor microenvironment (TME), DCs serve as the sentinels of the immune system, capable of directing both the activation and phenotype of tumor antigen-specific T-cell populations (1). This vital role in the generation of tumor immunity makes the DC a strategic focus for the evolution of cancer immune evasion mechanisms (2). This has been highlighted by studies in ovarian and prostate cancer suggesting that tumor-associated DCs acquire the capacity to actively tolerize the local immune microenvironment by promoting regulatory T-cell (Treg) development (3, 4). The tumor-derived signals and molecular mechanisms involved in DC tolerization within the TME remain poorly characterized. Data presented to date, however, suggest that the immune regulatory enzyme, indoleamine 2,3-dioxygenase-1 (IDO), likely contributes to this process (5). IDO catalyzes the degradation of the essential amino acid tryptophan into the kynurenines (6). While tryptophan depletion dampens T-cell proliferation, the generation of kynurenine drives the differentiation of Tregs (7). Although previous work has identified several stimuli that induce IDO, the critical signals that direct IDO expression and activity in the TME remain unknown (8).

Recent developments in melanoma immunotherapy with anti-CTLA-4 and anti-PD-1 monoclonal antibody (mAb) therapies have demonstrated the importance of the immune system in regulating melanoma progression and further illustrates the significance of immune regulatory pathways in cancer immunobiology (9, 10). Although these “checkpoint” inhibitors have shown impressive clinical results, many patients with advanced cancer remain refractory
to this treatment strategy. One potential explanation for these clinical outcomes is the evolution of tumor immune evasion mechanisms that target local DC populations. Therefore, an improved understanding of the alterations that these antigen-presenting cells (APC) undergo within the TME is vital for the development of novel strategies that may enhance our current immunotherapy arsenal.

The β-catenin signaling pathway plays an important role in DC-mediated immune suppression both *in vitro* and *in vivo* (11, 12). A more recent study has shown that Wnt5a promotes *in vitro* differentiation of human monocytes into a tolerized DC population. Further work supports a role for Wnt ligands in DC-mediated *in vitro* Treg differentiation in the presence of TGF-β (13, 14). Together, these findings suggest that the Wnt-β-catenin signaling pathway promotes DC tolerization; however the physiologic stimuli regulating this signaling pathway in the context of cancer remains unclear. In addition, it is not known whether this signaling pathway is associated with the activity of the IDO immune regulatory enzyme or whether expression of β-catenin target genes in DCs may reflect an immune tolerant TME. Finally, strategies to manipulate this pathway to enhance antitumor immunity have yet to be investigated.

Soluble Wnt ligands in the TME drive melanoma development (15) and Wnt5a promotes melanoma metastasis (16-18). While studies have shown evidence of a tumor intrinsic β-catenin signaling pathway in driving melanoma progression, these data cannot exclude a potential oncogenic role for paracrine Wnt-mediated signaling within the stroma of the TME (19). Previous studies have described the establishment of Wnt ligand concentration gradients driving short-range paracrine signaling, however recent data have indicated longer-distance
paracrine signaling in some biologic contexts (20–22). Further studies have also demonstrated a role for Wnt paracrine signaling mechanisms in the development of some cancers (23, 24).

In this work, we investigate the melanoma-derived signals regulating the β-catenin signaling pathway in local DCs both in vitro and in vivo, we characterize a key element involved in DC β-catenin-dependent Treg differentiation, and we explore the translational implications of this DC-specific signaling pathway in the development of more effective cancer immunotherapy strategies.

Methods

Mice. BALB/c(H-2<sup>d</sup>), C57BL/6(H-2<sup>b</sup>), Tyr::CreER;Braf<sup>CA</sup>;Pten<sup>lox/lox</sup>(H-2<sup>b</sup>), Tg(TCF/Lef1-HIST1H2BB/EGFP)61Hadj/J(H-2<sup>b</sup>), and B6.129-Indo<sup>im1Alm</sup>/J(H-2<sup>b</sup>) mice were purchased from Jackson Labs. FoxP3-mRFP mice (H-2<sup>b</sup>) were a gift from H.K. Lyerly (Duke University). All experiments performed under an IACUC-approved protocol.

Cell Lines. The B16/F10, COS7, and 293T cell lines were obtained from ATCC and maintained in DMEM, 10% FBS and the HEK293-LEF1/TCF-luciferase reporter stable cell line (a gift of L. Ring, Ludwig-Maximilians-Universität) in DMEM, 10% FBS with 1 mg/mL G418 (25). The human melanoma cell line WM266 was obtained from the Duke Cell Culture Facility and maintained in MEM, pyruvate, NEAA, and 10% FBS. The Hu-175, Hu-422, Hu-424, and Hu-451 human melanoma cell lines were derived from human melanoma explants at DUMC (26). The Braf<sup>V600E</sup>PTEN<sup>−/−</sup> cell line was generated by spontaneous immortalization of a resected Tyr::CreER;Braf<sup>CA</sup>;Pten<sup>lox/lox</sup> tumor and cultured in RPMI, 10% FBS. The DC2.4 cell line (a gift of K. Rock, University of Massachusetts) was maintained in RPMI, 10% FBS.
Stable Cell Line Generation. B16-PORCN\textsuperscript{KD}/B16-NTC cell lines were generated using a PORCN-targeted shRNA-expressing or control shRNA-expressing lentivirus followed by puromycin selection. The BRAF\textsuperscript{V600E}PTEN\textsuperscript{—/-}-Wnt5a\textsuperscript{KD} and BRAF\textsuperscript{V600E}PTEN\textsuperscript{—/-}-NTC stable cell lines were generated similarly.

Murine Bone Marrow-derived Dendritic Cells (BMDC) were generated as previously described (27) and purified using CD11c microbeads (Miltenyi Biotec) according to the manufacturer’s protocol.

Human Monocyte-derived Dendritic Cells were generated as previously described (28).

Murine Cell Isolation. Tissues were resected and mechanically disaggregated. Spleens were further digested with collagenase D (Roche) and centrifuged on a M-lympholyte gradient (Cedarlane). Tumors were digested with collagenase IV, hyaluronidase, and deoxyribonuclease (Sigma) at 37°C for 3 hours. DCs were purified using CD11c microbeads and naïve CD4\textsuperscript{+} T cells isolated with a negative selection kit (Miltenyi Biotec).

Reagents. β-catenin and PORCN(15G12.1) antibodies were purchased from Millipore, IDO(mIDO-48) from Biolegend, HA(HA11) from Covance. PTEN, p-ERK, t-ERK and Stat1 were purchased from Cell Signaling and β-actin (AC-15) from Sigma. The following antibodies were purchased from BD Pharmingen: I-A\textsuperscript{d}(2G9), CCR7(4B12), CD3(145-2C11), CD4(RM4-5), CD8(53-6.7), CD11c(HL3), CD16/CD32(2.4G2), CD40(3/23), CD45(30-F11), CD80(16-10A1), CD86(GL1), FoxP3(MF23), and PD-L1(MIH5). Anti-GFP-AlexaFluor488 antibody were purchased from Invitrogen, Wnt5a from R&D Systems, and gp100(EP4863(2)) from Abcam. PCNA(F-2), S100β, TRP1(H-90), TRP2(D-18) and tyrosinase(H-109) antibodies were purchased from Santa Cruz. Wnt3a, Wnt5a and IFN\gamma ligands were purchased from R&D. Both the XAV939 and 1-MT
inhibitors were purchased from Sigma-Aldrich: D/L-1-MT for in vitro studies; D-1-MT for in vivo studies. Hydroxyamidine INCBO24360 analogue (cat# 205493) (29) and C59 inhibitor were purchased from MedKoo Biosciences and Cellagen Technology, respectively.

ChIP Assays. Purified DCs were treated with Wnt3a, Wnt5a, or IFNγ, and chromatin immunoprecipitation assays (ChIPs) were performed using SimpleChIP Plus Enzymatic Chromatin IP Kit (Cell Signaling).

Luciferase Assays. COS7 cells were transfected with the pGL2-mIDO1 firefly luciferase reporter plasmid (a gift of A. Muller, Lankenau Institute for Medical Research), treated with Wnt3a, Wnt5a, and IFNγ. IDO promoter activation was analyzed using the Dual-Glo Luciferase Assay System. Wnt activity in CM was assayed with a HEK293-LEF1/TCF-luciferase reporter stable cell line and the Pierce Gaussia Luciferase Glow Assay Kit (ThermoScientific).

Proliferation Assays. B16-NTC, B16-PORCN^{KD}, BRAF^{V600E-PTEN^{f/-}}-Wnt5a^{KD}, and BRAF^{V600E-PTEN^{f/-}}-NTC cell lines were pulsed with [³H]thymidine (1 μCi, Perkin Elmer) for 24 and 48 hrs.

ELISAs. ELISAs were performed according to manufacturer’s instructions using a mouse Wnt5a ELISA kit (Cusabio Life Science), a mouse IL10 ELISA kit (R&D), and a TGFβ ELISA kit (R&D).

Flow Cytometry. Cells were stained according to standard protocols and analyzed using a BD FACSCanto II or LSRII. FoxP3 staining was performed according to manufacturer’s instructions (BD Pharmingen). Tumor antigen-specific T-cell populations in B16/F10 melanoma-bearing syngeneic mice were quantified by flow cytometry using an APC-labelled H-2K^b-TRP2 dextramer (Immudex).

Measurement of Intracellular Ca^{2+}. DC intracellular Ca^{2+} was measured using a Nikon Ti-S inverted microscope as previously prescribed (30).
**IDO Enzymatic Assay.** IDO enzymatic activity was measured by high-performance liquid chromatography (HPLC) as previously described (31).

**In vitro Treg Assay.** DCs (H-2d) were treated with Wnt3a or Wnt5a for 18-48 hr at 37°C, washed, and re-plated at a 10:1, 5:1, or 1:1 T-cell:DC ratio with purified allogeneic (H-2b) naïve splenic CD4+ T cells. These cultures were incubated for 6 days, harvested, and CD4+FoxP3+ T-cell populations quantified by flow cytometry.

**In vivo Treg Assay.** DCs or IDO1−/− DCs (H-2b) were treated with Wnt5a or vehicle control for 48 hr at 37°C, washed, and delivered by intradermal injection into the footpad of FoxP3-mRFP transgenic mice. Inguinal lymph nodes were harvested 4 days later and analyzed by flow cytometry for CD4+mRFP+ Tregs.

**Quantitative RT-PCR (qrt-PCR).** RNA was isolated using the RNeasy Mini Kit (Qiagen) and reverse-transcribed using the iScript cDNA Synthesis Kit (BioRad) before quantitative RT-PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) on an AB7500 Real-Time PCR Instrument. Primer sequences are provided in **Supplementary Table 1**. Relative transcript levels were calculated according to the $2^{-\Delta\Delta Ct}$ method; data normalized to GAPDH.

**PCR-based Quantitation of Melanoma LN Metastasis.** Genomic DNA was isolated from whole lymph node tissues and levels of puromycin N-acetyl transferase were measured as a surrogate marker of melanoma metastasis based on semi-quantitative PCR using the Apex Hot Start 2.0 Master Mix (Genesee).

**Microarray Analysis.** Microarray data sets publicly available on the Oncomine Cancer Profiling Database (Oncomine 4.4) were used to investigate Wnt ligand and FoxP3 expression in human benign nevi and melanoma tissues.
**In vivo Tumor Experiments.** B16/F10 (15 x 10^3), B16-NTC/B16-PORCN^KD (1 x 10^5), and BRAF^V600E^PTEN^-/-Wnt5a^KD/BRAF^V600E^PTEN^-/-NTC (50 x 10^5) cells were implanted into syngeneic C57BL/6 mice and tumor growth monitored by caliper measurement. Tumor and TDLN tissues were resected between days 14-21. *Tyr::CreER;BrafCA;Pten^lox/lox* transgenic mice were treated with topical 4-HT (Sigma, 20 mM) for 2 days to induce primary melanoma development. 5 mg/kg/day C59 was administered daily by oral gavage and anti-CTLA-4 mAb or hamster IgG1 isotype control (BioXCell) was delivered every 3 days by intraperitoneal injection (100 µg/dose). D-1-MT was administered twice daily by oral gavage (200 mg/kg/dose). The IDO1-selective hydroxyamidine inhibitor was delivered by subcutaneous injection twice daily (75 mg/kg/dose).

**Immunohistochemistry/Immunofluorescence.** Paraffin-embedded tissues were processed and stained following standard protocols and imaged with a Zeiss CLSM 700 confocal microscope.

**Statistical Analysis.** Statistical analysis performed using GraphPad Prism 6.0. Mann-Whitney U-tests used in animal experiments. Multiple comparison analyses were performed using non-parametric one-way ANOVA and two-way ANOVA followed by post-hoc multi-comparison testing. Wnt5a expression levels were correlated with FoxP3 expression levels based on a non-parametric Spearman correlation calculation. The remainder of statistical analyses utilized an unpaired *t*-test. All statistical tests were two tailed and used an *α* of 0.05. All error bars represent S.E.M.

**Human LN DC Isolation.** Eight patients were consented and accrued onto the LN study approved by the DUMC Institutional Review Board. Lymphoscintigraphy identified the sentinel LN for harvest and biopsy. Single-cell suspensions were generated from a sample of sentinel LN tissue using a GentleMACS dissociator (Miltenyi biotec) and DC populations were purified using
magnetic microbeads (Miltenyi biotec). Cell purity was verified by flow cytometry and total RNA purification from each cell population was performed using an RNeasy Micro Kit (Qiagen).

Results

**Human Melanoma Induces Paracrine β-catenin Signaling Activation in DCs.**

Based on previous data implicating the β-catenin transcription factor in the induction of DC tolerization (11, 12), we conducted an *in silico* expression analysis of Wnt ligands and the Treg marker, FoxP3, using a human melanoma microarray dataset. We confirmed that several Wnt ligands are upregulated in melanoma compared with normal melanocytes ([Supplementary Fig 1](#)) and found a significant correlation in Wnt5a and FoxP3 expression in human melanoma tissues ([Fig 1A](#)).

Both pre-clinical and clinical data suggest that Wnt5a promotes melanoma metastasis (16, 17). Based on these findings and our recent data, we hypothesized that melanoma-derived soluble Wnt5a may promote DC tolerization via a paracrine β-catenin-dependent signaling pathway and drive FoxP3+ Treg differentiation within the TME ([Fig 1B](#)). To initially examine this hypothesis, we harvested and co-cultured the conditioned media (CM) of human melanoma-derived cell lines with COS7 cells transfected with a TCF/LEF1-GFP reporter construct. Subsequent studies consistently showed human melanoma-derived CM to activate the β-catenin signaling pathway in these reporter cell lines ([Fig 1C](#)). These findings were further corroborated by co-culturing CM harvested from several additional human melanoma cell lines with a stable HEK293 reporter expressing a TCF/LEF1-responsive promoter driving Gaussian luciferase expression ([Fig 1D](#)). Additional studies showed a human metastatic melanoma cell
line (WM266) to express higher levels of Wnt5a than a primary human melanoma cell line (WM115) and that these levels correlated with stabilization of β-catenin in human DCs (Fig 1E). These data suggest that soluble Wnt ligands, including Wnt5a, may be capable of impacting local DC function in the human melanoma microenvironment.

**Melanoma-derived Wnt5a Induces β-catenin Signaling Pathway Activation in DCs In vitro and In vivo.**

Based on these data, we explored Wnt-dependent paracrine signaling networks in a murine melanoma model. We initially confirmed that B16 melanoma-derived CM induces the activation of a TCF/LEF1-luciferase reporter cell line and stabilizes primary DC β-catenin levels in vitro (Supplementary Fig 2). To study this Wnt-mediated paracrine signaling pathway in vivo, we utilized the Tyr::CreER;BrafCA;Ptenlox/lox transgenic melanoma mouse model which closely recapitulates human melanomas by expressing a melanocyte-specific BRAFV600E activation mutation in the absence of the PTEN tumor suppressor (Supplementary Fig 3A,B) (19). To determine if Wnt ligands expressed by this melanoma model alter local DC signaling pathways, we analyzed the expression of a panel of β-catenin target genes in purified tumor-infiltrating CD45⁺F4/80⁻CD11c⁺IAb⁺ DC (TIDC) populations using qrt-PCR and compared these expression levels with control BMDCs isolated from non-tumor-bearing mice (Supplementary Fig 4A, B). These experiments showed TIDCs to express enhanced levels of the β-catenin-responsive genes Axin-2, Ccnd1, C-myc, and Tcf-7 relative to control BMDCs (Fig 2A). To determine if soluble Wnt ligands expressed by this in vivo melanoma model modulate β-catenin signaling in DCs residing within nearby TDLN tissues, we harvested the ipsilateral brachial, and axillary TDLNs from
tumor-bearing Tyr::CreER;BrafCA;Ptenloxp/lox mice and purified CD11c+ DCs for further analysis (Supplementary Fig 4C). These studies showed enhanced levels of β-catenin responsive genes in TDLN DCs relative to distant LN DCs and LN DCs harvested from non-tumor-bearing mice (Fig 2B). These data suggest that melanoma-expressed soluble Wnt ligands regulate locoregional DCs in vivo. To further confirm these findings, we generated an immortalized cell line derived from the resected tumor tissue of Tyr::CreER;BrafCA;Ptenloxp/lox mice (Supplementary Fig 5) and implanted this BRAF^V600EPTEN^-/- cell line into syngeneic Tg(TCF/Lef1-HIST1H2BB/EGFP)61Hadj/J transgenic β-catenin-dependent GFP reporter mice (32). After primary tumor development, both tumor tissues and TDLN tissues were resected and the DC populations were evaluated for GFP expression by flow cytometry and confocal microscopy. These data revealed that a significant portion of TIDCs and TDLN DCs exhibited evidence of β-catenin signaling activation (Fig 2C-E).

Similar to human melanoma, Tyr::CreER;BrafCA;Ptenloxp/lox melanoma tumors express high levels of Wnt5a (Supplementary Fig 3C,D). To determine the contribution of Wnt5a in regulating the DC β-catenin signaling pathway observed above, we genetically silenced Wnt5a expression in the BRAF^V600EPTEN^-/- melanoma cell line (BRAF^V600EPTEN^-/-;Wnt5aKD) and implanted this line into the β-catenin-dependent GFP reporter mouse model to determine the specific impact of Wnt5a on β-catenin signaling within nearby stromal tissues (Supplementary Fig 7A,B). Using confocal microscopy, we determined BRAF^V600EPTEN^-/-;Wnt5aKD tumors to be associated with a significant reduction in paracrine β-catenin-dependent signaling within the TME (Fig 2F).
Taken together, these data suggest that melanoma-derived Wnt5a induces paracrine β-catenin signaling activity in DCs both in vitro and in vivo.

**Melanoma-derived Wnt5a Conditions DCs to Promote Regulatory T-cell Development.**

Flow cytometry analysis of TDLNs resected from Tyr::CreER;BrafCA;Ptenlox/lox mice bearing Wnt5a-expressing melanoma tumors indicate that these tissues harbor increased levels of CD4^+FoxP3^+ Tregs relative to LN tissues resected from non-tumor-bearing mice (Fig 3A). In addition, BRAF<sup>V600E</sup>PTEN<sup>+/−</sup>-Wnt5a<sup>KD</sup> tumors were noted to be associated with reduced numbers of TDLN Tregs relative to BRAF<sup>V600E</sup>PTEN<sup>+/−</sup>-NTC control tumors further supporting an association between Wnt5a-expressing melanomas and local Treg populations (Fig 3B). Our data in human melanoma tissues also suggest that Wnt5a may condition DCs to drive Treg development, a characteristic consistent with the process of DC tolerization (Fig 1).

To address this question directly, we pre-treated purified BMDCs with recombinant Wnt5a and several other known DC stimuli as controls. These DCs were harvested and co-cultured with purified splenic CD4^+ T cells and then analyzed by flow cytometry to quantify CD4^+FoxP3^+ Tregs (Fig 3C, Supplementary Fig 8). In contrast to other stimuli, pre-conditioning DCs with Wnt5a significantly enhanced their ability to expand Tregs in vitro. To demonstrate that Wnt5a is the primary mediator of DC tolerization and Treg generation in the melanoma microenvironment, we co-cultured DCs with CM harvested from the BRAF<sup>V600E</sup>PTEN<sup>+/−</sup>-Wnt5a<sup>KD</sup> and BRAF<sup>V600E</sup>PTEN<sup>+/−</sup>-NTC melanoma cell lines and repeated the above in vitro Treg assay using naïve CD4^+CD62L^+ T cells (Fig 3D). These data show that melanoma-expressed soluble Wnt5a is critical for conditioning DCs to drive Treg generation in vitro. To verify that Wnt5a is capable of
also inducing DC-mediated Treg differentiation in situ, we purified DCs from resected BRAF<sup>V600E</sup>PTEN<sup>−/−</sup>-Wnt5a<sup>KD</sup> and BRAF<sup>V600E</sup>PTEN<sup>−/−</sup>-NTC tumor tissues for in vitro Treg assays which further validated Wnt5a as an important mediator of DC-mediated Treg generation in vivo (Fig 3E).

**Wnt5a Stimulates β-catenin-dependent Durable Indoleamine 2,3-dioxygenase-1 Expression by DCs.**

To better understand the mechanism by which Wnt5a licenses DCs to promote Treg expansion, we analyzed the phenotype of Wnt5a-treated BMDCs by flow cytometry and found no significant effect of Wnt5a on class II MHC, CD40, CD80, CD86, CCR7, or PD-L1 surface expression (Supplementary Fig 9A). Further ELISA studies also did not show Wnt5a inducing any significant DC expression of IL10 or TGFβ at various time points (Supplementary Fig 9B). However, noting that other investigators have shown the IDO1 promoter to comprising LEF1 binding elements (33), we purified TIDCs and TDLN-derived DCs from Tyr::CreER;Braf<sup>C58</sup>;Pten<sup>lox/lox</sup> transgenic mice and found these DCs to express elevated levels of IDO relative to LN-derived DCs purified from non-tumor-bearing hosts (Fig 4A). To determine if Wnt5a contributed to the differences in DC IDO expression observed in these experiments, DCs were isolated from BRAF<sup>V600E</sup>PTEN<sup>−/−</sup>-Wnt5a<sup>KD</sup> and BRAF<sup>V600E</sup>PTEN<sup>−/−</sup>-NTC control tumors and analyzed for IDO expression. Indeed, these assays revealed diminished IDO expression in DCs harvested from the BRAF<sup>V600E</sup>PTEN<sup>−/−</sup>-Wnt5a<sup>KD</sup> tumors suggesting that Wnt5a regulates DC-dependent IDO expression in vivo (Fig 4B). These findings prompted us to treat purified BMDCs with Wnt5a and probe for IDO expression by Western blot (Fig 4C). These experiments showed
lithium chloride (LiCl), a GSK-3β inhibitor, and both Wnt5a and Wnt3a to promote the upregulation of IDO expression. To confirm these findings, we transfected COS7 cells with a previously engineered luciferase reporter construct downstream of the IDO promoter sequence and treated these cells with Wnt5a, Wnt3a, and IFNγ as a positive control (34). Consistent with the above data in primary BMDCs, both Wnt5a and Wnt3a induced IDO promoter activity to levels exceeding those induced by IFNγ (Fig 4D). Since other investigators have shown previously that IDO expression and enzymatic activity may be differentially regulated (31, 35), we investigated if Wnt-stimulation of DCs also enhanced IDO enzymatic activity. Using an HPLC-based approach to detect accumulation of the metabolic product kynurenine, we found Wnt5a to significantly stimulate the enzymatic function of IDO (Fig 4E, Supplementary Fig 10A).

Consistent with previous data, our findings suggest that the β-catenin transcription factor is important for regulating the DC tolerization program. While previous studies have shown that Wnt5a primarily signals via a β-catenin-independent non-canonical signaling pathway (36), more recent work has suggested that Wnt5a-mediated stimulation of the canonical β-catenin pathway may be dependent upon the receptor repertoire of the cell (37, 38). Indeed, our qrt-PCR expression analysis of the known Wnt ligand receptors/co-receptors in DCs demonstrates a profile that is compatible with Wnt5a-mediated activation of the β-catenin pathway (Supplementary Fig 10B,C). In addition, our studies did not show any evidence of Wnt5a induces the non-canonical Ca²⁺-dependent signaling pathway in DCs (Supplementary Fig 10D). To further investigate the role of β-catenin in Wnt5a-mediated stimulation of IDO in DCs, we performed Wnt5a stimulation assays utilizing the β-catenin inhibitor, XAV939 (39). This work showed XAV939 to effectively inhibit Wnt5a- and Wnt3a-dependent induction of IDO.
expression (Fig 4C). We then validated the role of Wnt5a-mediated β-catenin signaling in the upregulation of IDO by performing ChIP assays demonstrating that both Wnt3a and Wnt5a induce β-catenin binding to the IDO promoter in BMDCs (Fig 4F). These findings were further supported by additional assays utilizing a myeloid DC line that we genetically silenced β-catenin (DC2.4-βcat^KD) and TCF/LEF1-EGFP reporter BMDCs (Fig 4G, Supplementary Fig 10E). Finally, we demonstrate Wnt5a to induce IDO expression in splenic plasmacytoid DCs suggesting that this is not a myeloid-specific phenomenon (Supplementary Fig 10F). Altogether, this work implicates β-catenin as playing an important role in Wnt5a-mediated upregulation of IDO expression in DCs.

To confirm that melanoma-derived Wnt5a stimulates DC IDO expression, we pre-incubated B16 melanoma cells with anti-Wnt5a antibody prior to co-culturing the CM with primary DCs. Subsequent Western blot analysis showed that Wnt5a blockade abrogated the expression of both IDO and β-catenin (Fig 4H). Similar data were obtained with recombinant soluble Fzd3 receptor (sFRP3) before co-incubation with primary BMDCs. However, neither anti-Wnt3a antibody nor sFRP2 impacted BMDC expression of IDO in this system (Supplementary Fig 11A,B). Since previous studies have reported that sFRP3 binds to Wnt5a with higher affinity than to Wnt3a, we conclude that melanoma-derived Wnt5a plays the dominant role in stimulating IDO expression by DCs (40, 41).

Interestingly, our Western blot data indicate that Wnt5a, relative to Wnt3a, stimulates prolonged IDO expression (Fig 4C). To investigate the kinetics of β-catenin activation by Wnt5a and Wnt3a, we treated BMDCs with these ligands and quantified β-catenin nuclear translocation at various time points by fluorescence microscopy (Supplementary Fig 11C).
Results from these experiments were consistent with those from our Western blot studies, indicating that Wnt5a-dependent induction of β-catenin in DCs was prolonged relative to that by Wnt3a stimulation. Previous work in human peripheral blood mononuclear cells has shown Wnt5a stimulates IFNγ expression (42). These data raised the possibility that Wnt3a- and/or Wnt5a-dependent induction of IDO expression in DCs was partially IFNγ-dependent. By repeating these in vitro stimulation assays in the presence of an anti-IFNγ blocking antibody, we determined that Wnt3a-mediated but not Wnt5a-mediated upregulation of IDO expression was dependent upon IFNγ (Supplementary Fig 12A). These findings are further supported by qrt-PCR analysis revealing that Wnt3a, but not Wnt5a, induced the expression of IFNγ by BMDCs (Supplementary Fig 12B).

In summary, these data suggest that Wnt5a upregulates the expression of the IDO immunoregulatory enzyme in DCs and that β-catenin is an important mediator of this signaling pathway. These results provide mechanistic evidence that IDO plays a significant role in β-catenin-dependent DC tolerogenesis.

**Wnt5a Conditions DC-mediated Regulatory T-cell Development via an IDO-dependent Mechanism both In vitro and In vivo.**

Based on our previous data, we sought to determine whether Wnt5a could promote DC-mediated Treg differentiation via IDO upregulation. Therefore, we performed in vitro Treg assays using purified naïve CD4⁺CD62L⁺ T cells in the presence and absence of the IDO inhibitor, 1-methyltryptophan (D,L-1-MT) (Fig 5A). In line with our previous findings, the use of D,L-1-MT
in these assays completely abolished Treg generation indicating that Wnt5a-conditioned DCs drive in vitro Treg differentiation in an IDO-dependent manner.

We previously noted that diminished levels of IDO expression in TIDCs correlated with a reduction in TDLN Treg populations in BRAF<sup>V600E</sup>PTEN<sup>−/−</sup>-Wnt5a<sup>KD</sup> tumors relative to BRAF<sup>V600E</sup>PTEN<sup>−/−</sup>-NTC tumor controls suggesting that DC-expressed IDO also played a role in Wnt5a-mediated Treg generation in vivo (Figs 3B, 4B). To confirm this hypothesis, we delivered untreated and Wnt5a-treated wild-type and IDO1<sup>−/−</sup> DCs into the footpad of FoxP3-mRFP transgenic mice and examined draining lymph node tissues for CD4<sup>+</sup>mRFP<sup>+</sup> Tregs by flow cytometry (Supplementary Fig 13). While Wnt5a-stimulated wild-type DCs reproducibly enhanced Treg generation in situ, this effect was eliminated when Wnt5a-stimulated IDO1<sup>−/−</sup> DCs were delivered, indicating that Wnt5a-dependent Treg generation relies upon DC expression of IDO in vivo (Fig 5B). Given the role of IDO in Wnt5a-mediated Treg generation, we also explored the role of the Wnt5a-DC IDO signaling axis in melanoma development in vivo. Using the BRAF<sup>V600E</sup>PTEN<sup>−/−</sup>-NTC and BRAF<sup>V600E</sup>PTEN<sup>−/−</sup>-Wnt5a<sup>KD</sup> melanoma model system, we determined that the genetic silencing of Wnt5a expression suppresses primary melanoma development and metastasis to draining lymph nodes (Fig 5C,D, Supplementary Fig 14). We then repeated these studies in the presence or absence of either D-1-MT or a hydroxyamidine selective IDO1 inhibitor and found that inhibition of IDO activity suppressed BRAF<sup>V600E</sup>PTEN<sup>−/−</sup> melanoma development only in the presence of Wnt5a, suggesting that the pro-tumorigenic properties of Wnt5a are dependent upon IDO enzymatic activity (Fig 5E). Overall, these results indicate that IDO is critical for the immune suppressive properties of Wnt5a in melanoma.
The PORCN A cyltransferase Enzyme Regulates Melanoma Wnt5a Secretion and Melanoma Progression In vivo.

The membrane-bound O-acyltransferase enzyme, PORCN, catalyzes the palmitoylation of the Wnt ligands, enabling their secretion and stimulation of nearby tissues (Fig 6A). Based on our cumulative data, we hypothesized that genetically silencing PORCN expression to block Wnt secretion in B16 melanoma would reverse immune tolerance and suppress tumor progression in vivo. To address this hypothesis, we engineered a stable B16 cell line genetically silenced for PORCN expression (B16-PORCN-KD) and a non-targeted control B16 cell line (B16-NTC) (Supplementary Fig 15A,B). After verifying silenced PORCN expression by qrt-PCR and Western blot analysis, we co-incubated the CM from both cell lines with the HEK293-LEF1/TCF-luciferase reporter as described above (Fig 6B). This experiment showed diminished luciferase activity when reporter cell lines were co-incubated with B16-PORCN-KD-derived CM. We then transfected these cell lines with a HA-tagged Wnt5a-expressing plasmid and probed for Wnt5a secretion by anti-HA immunoprecipitation and Western blot analysis to demonstrate that the B16-PORCN-KD cell line was not able to effectively secrete the Wnt5a ligand, an effect that was recapitulated with pharmacologic inhibition of PORCN activity (Fig 6C). Wnt5a secretion was further confirmed to be impaired in the B16-PORCN-KD cell line based on ELISA (Fig 6D). We next sought to determine if the inhibition of Wnt secretion by the B16-PORCN-KD cell line impacted cell proliferation or apoptosis however we were unable to appreciate any significant alterations in these cellular processes (Supplementary Fig 15C-E). Despite these findings, further in vivo tumor studies demonstrated that B16-PORCN-KD tumors exhibited diminished growth relative to the B16-NTC control tumors (Fig 6E). Furthermore, flow cytometry analysis
showed that B16-PORCN-KD tumors harbored increased numbers of infiltrating CD8+ T-cells (Fig 6F). This was similar to our observations in the BRAFV600E-PTEN−/− tumor model in which the genetic silencing of Wnt5a expression is also associated with increased numbers of infiltrating CD8+ T cells (Supplementary Fig 15F). Altogether, these data suggest that PORCN-mediated Wnt5a secretion by melanomas generates an immunotolerant TME that ultimately allows tumor progression.

**C59 Inhibition of the PORCN-Wnt-β-catenin Signaling Pathway Synergizes with anti-CTLA-4 Antibody Immunotherapy in the B16 Melanoma Model.**

Based on our previous work, we hypothesized that inhibiting PORCN enzymatic activity could reverse melanoma induction of the DC β-catenin-IDO signaling pathway. To test this hypothesis, we treated several murine and human melanoma cell lines with a soluble PORCN inhibitor, C59, and co-cultured the resulting CM with the stable HEK293-LEF1/TCF-luciferase reporter cell line. Indeed, treatment with C59 diminished Wnt-mediated β-catenin-dependent paracrine signaling in each melanoma cell line (Supplementary Fig 16, Fig 7A). To further determine if the C59 inhibitor is capable of modulating IDO expression by BMDCs in vitro, we harvested and co-cultured the CM of C59-treated B16/F10 melanoma cells with purified BMDCs and performed Western blot analysis of DC-expressed IDO and cytosolic β-catenin protein levels (Fig 7B). Consistent with our previous findings, these data show the C59 PORCN inhibitor to coordinately suppress IDO and β-catenin in BMDCs.

We, therefore, investigated the ability of C59 to augment the antitumor effects of the anti-CTLA-4 mAb checkpoint inhibitor. Since anti-CTLA-4 mAb targets T cells, we hypothesized
that this approach could augment anti-CTLA-4 efficacy by reversing tumor-mediated tolerization of local DC populations that orchestrate tumor antigen-specific T-cell activation. We first confirmed that oral dosing of the C59 inhibitor suppressed β-catenin stabilization in this murine tumor model by whole tissue Western blot (Supplementary Fig 17A). We then implanted B16/F10 melanoma cells into syngeneic C57BL/6 hosts, treated these mice with C59 or a vehicle control daily by oral gavage and administered anti-CTLA-4 mAb or an IgG isotype control by intra-peritoneal injection every three days. Interestingly, we found C59 treatment to significantly suppress B16 melanoma progression when administered in combination with anti-CTLA-4 mAb therapy, suggesting a synergistic enhancement in antitumor immunity (Fig 7C). This was further supported by flow cytometry analysis of tumor-infiltrating T cells which showed evidence of enhanced CD8+ T-cell activation based on 41BB surface expression and an augmented population of tumor antigen-specific CD8+ T cells based on Kb-TRP2 dextramer analysis (Fig 7D, Supplementary Fig 17B). This work provides additional in vivo evidence that supports the notion that the Wnt-β-catenin pathway modulates the immune microenvironment in melanoma by conditioning local DCs to suppress CD8+ T-cell activation (Fig 7E). In addition, these data introduce this pathway as a potential pharmacologic target in tumor immunotherapy.

**Sentinel LN DC expression of IDO and β-catenin Target Genes is Associated with Melanoma Disease Burden.**

The data presented above suggest that Wnt5a-induction of the β-catenin pathway promotes DC tolerization. This implies that a melanoma microenvironment comprising DC populations that
exhibit a gene expression signature indicative of β-catenin signaling pathway activation is more likely to be associated with a higher tumor burden and potentially diminished relapse-free survival. We have initiated a program to investigate sentinel LN DC gene expression signatures as possible biomarkers for melanoma behavior and treatment response. In the initial panel of patients undergoing a staging sentinel LN biopsy, we isolated both myeloid and plasmacytoid DC populations and purified total RNA from these cells for downstream qrt-PCR analysis (Supplementary Fig 18A). Using this approach, we have identified a relationship between melanoma disease burden within the draining LN bed and elevated levels of DC TCF7, IDO1, and CCND1 expression (Fig 7F, Supplementary Fig 18B). Although these data require confirmation in a larger patient cohort, it is interesting that the only patient with metastatic progression exhibited the most significant elevations in sentinel LN DC-specific expression of these β-catenin-dependent genes. These findings support our previous data and indicate that LN DC populations may be a useful resource for biomarker development in tumor immunotherapy.

Discussion

As emerging data substantiated the role of the DC in orchestrating tumor-targeted T-cell activation, investigators noted tumor-derived mechanisms interfering with DC maturation (43, 44). Our understanding of these mechanisms of immune evasion has expanded to include the active induction of a potent DC tolerization program (3, 4). Tolerized DCs establish a site of immune privilege by driving Treg differentiation and propagating regional immune suppression. However, the signals that induce this DC tolerization process are unclear.
Here we demonstrate that melanoma secretion of Wnt5a conditions local DC populations to promote Treg development. We show that this process of DC-dependent Treg differentiation is dependent upon a novel \( \beta \)-catenin-IDO signaling pathway that is upregulated within DC populations that reside within the TME. While our data suggest that the Wnt3a ligand is capable of inducing signals on a transient time scale and is IFN\( \gamma \)-dependent, the Wnt5a-induced signal is more durable and is independent of IFN\( \gamma \) signaling. Interestingly, these findings are similar to that reported for the differential signaling kinetics between IFN\( \gamma \)- and TGF\( \beta \)-induced activation of IDO in plasmacytoid DCs and may partially explain the differences observed between Wnt3a- and Wnt5a-induced DC-mediated Treg differentiation (31).

Using both genetic ablation and pharmacologic inhibition, we demonstrate melanoma-derived Wnt5a promotes tumor growth in vivo and that this correlates with the inhibition of antitumor adaptive T-cell responses. While only modest antitumor activity is observed as a single agent, the C59 PORCN inhibitor synergistically suppressed B16 melanoma development when combined with anti-CTLA-4 blockade in syngeneic mice. The therapeutic effect of C59 was unlikely to be secondary to a direct antitumor mechanism as shRNA-targeted silencing of PORCN showed no significant impact on tumor cell proliferation or apoptosis either in vitro or in vivo. In addition, these inhibitory effects closely correlated with activation of tumor-infiltrating CD8\(^+\) T cells and expansion of tumor antigen-specific CD8\(^+\) T-cell populations. Notably, this relationship is similar to what we have previously observed when combining the LY2157299 type I TGF\( \beta \) receptor serine/threonine kinase inhibitor, another agent capable of modulating DC function, with anti-CTLA-4 antibody therapy in the \textit{Tyr::CreER;Braf\textsuperscript{CA};Pten\textsuperscript{lox/lox}}
transgenic melanoma model in which we also observed a synergistic induction in antitumor immunity (45, 46).

Oderup and colleagues showed that Wnt3a and Wnt5a differentially induced DCs to express the immunosuppressive cytokines, TGFβ, IL10, and VEGF while augmenting TGFβ induction of Treg differentiation in vitro (14). However, we were unable to demonstrate Wnt3a- or Wnt5a-induced immunosuppressive cytokine expression by DCs and our data suggest that Wnt5a alone is capable of driving DC-dependent Treg differentiation. Oderup and colleagues further show that only Wnt3a and not Wnt5a signals via a β-catenin signaling pathway in DCs. However, using a variety of experimental approaches, our work supports a role for β-catenin in Wnt5a-induced DC IDO expression. Indeed, our studies show no evidence that Wnt5a signals via a calcium-dependent non-canonical pathway in DCs. The reasons for these discrepancies may include the use of supra-physiologic concentrations of Wnt3a and Wnt5a and relatively short Wnt-stimulation times in several assays reported by Oderup and colleagues (47).

We demonstrate that melanoma-derived Wnt5a enables DCs to upregulate potent IDO activity once they encounter antigen-specific CD4⁺ T cells in nearby TDLN tissues, allowing for local Treg differentiation and promotion of immune tolerance. This interpretation expands on the work of Spranger and colleagues, who suggest that the induction of IDO activity in the TME is primarily a negative feedback response from IFNγ-expressing T cells (48). Here, we describe a mechanism whereby melanomas co-opt IDO function and actively manipulate their immune microenvironment to subvert host immunity, indicating that IDO induction is not simply part of a negative feedback regulatory system. The evolution of such a mechanism by developing cancers may, in fact, be required for disease progression in certain contexts.
Our current findings also suggest that reversing Wnt-mediated paracrine signaling potentiates the induction of tumor antigen-specific immune responses elicited by other immunotherapeutic strategies. Given that IDO has been implicated in promoting resistance to anti-CTLA-4 Ab blockade in murine melanoma models, the reversal of this pathway would be expected to augment anti-CTLA-4 Ab immunotherapy (49). Indeed, our data demonstrate the inhibition of Wnt signaling synergistically enhances the antitumor effect of anti-CTLA-4 mAb therapy in B16 melanoma. Although various strategies to inhibit the Wnt-β-catenin pathway are under investigation, targeting the membrane-bound O-acyl transferase, PORCN, is a promising approach in light of the specificity of these inhibitors (50). Indeed, pre-clinical studies have demonstrated PORCN inhibitors to suppress tumor growth with no detectable adverse effects and early phase clinical trials investigating the LGK974 PORCN inhibitor are ongoing (NCT01351103) (51, 52).

Although based on a limited number of patients, our data suggest a relationship between the activation of the β-catenin signaling pathway specifically within sentinel LN DCs and melanoma disease burden and limited progression-free survival. While additional studies are necessary, these data suggest that Wnt5a-mediated immune suppression is a mechanism that is relevant in human melanoma and that tumor-mediated alteration of the LN microenvironment is an important component of tumor-mediated immune evasion.

Using both an autochthonous melanoma model and specimens derived from patients with advanced melanoma, we demonstrate that the induction of IDO activity is a critical component of the Wnt-β-catenin–dependent DC tolerization program. Our work further reveals that melanomas are capable of generating a site of immune privilege by manipulating the β-
catenin signaling pathway in local DCs via the secretion of Wnt5a. These findings are consistent with data indicating that DC populations within the TME exhibit elevated levels of activated β-catenin and that this pathway drives Treg development in a retinoic acid-dependent manner (53). Furthermore, we provide data supporting a pharmacologic strategy for reversing this mechanism of immune evasion and demonstrate this approach to synergistically enhance the efficacy of a clinically relevant immune checkpoint inhibitor. Finally, we show evidence that sentinel LN-derived DCs may offer unique insight into the immune microenvironment while also serving as a potential resource for biomarker development in tumor immunotherapy.

Acknowledgments

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Figure Legends.

Figure 1. Human Melanoma Induces Paracrine β-catenin Signaling Activation in DCs in vitro.

A. Wnt5a and FoxP3 gene expression analysis in human melanoma. Riker Dataset, Oncomine 4.4. P-value, non-parametric Spearman correlation calculation. B. left, Wnt-β-catenin canonical signaling pathway. right, Hypothesis: Wnt5a-conditioned DCs promote Treg differentiation. C. Human melanoma CM co-incubated with TCF/LEF1-GFP-transfected COS7 reporter cell line and analyzed by flow cytometry (top) and β-catenin Western blot (bottom). D. Human melanoma CMs co-incubated with a 293T-TCF/LEF1-luciferase reporter cell line. P-value, one-way ANOVA.
E. left, Wnt5a ELISA of WM115 and WM266 CM. right, WM115 and WM266 CM co-incubated with human monocyte-derived DCs and analyzed by β-catenin Western blot. *P<0.05, ***P<0.0005.

Figure 2. Murine Melanomas Induce Paracrine β-catenin Signaling Activation in Tumor and TDLN DCs in vivo. A. qrt-PCR of β-catenin target gene expression by TIDCs isolated from Tyr::CreER;BrafCA;Ptenlox/lox primary melanomas relative to BMDCs derived from non-tumor-bearing mice. P-value, two-way ANOVA. B. qrt-PCR of β-catenin target gene expression by DCs isolated from TDLNs from melanoma-bearing Tyr::CreER;BrafCA;Ptenlox/lox mice compared to distant LNs and LNs from non-tumor-bearing cre- mice. P-value, one-way ANOVA. C. Left, flow cytometry analysis of GFP+CD11c+ DCs from TCF/Lef1-EGFP reporter mice. Right, confocal microscopy of a GFP+CD11c+ DC isolated from a BRAFV600EPTEN+/− primary melanoma. WT, wild type. D. GFP confocal microscopy of BRAFV600EPTEN+/− melanoma tissue resected from a TCF/Lef1-EGFP reporter mouse. 100x. E. Left, GFP confocal microscopy of TDLN tissue resected from TCF/Lef1-EGFP reporter mice. Center, TDLN tissue resected from WT BRAFV600EPTEN+/− melanoma-bearing mice. Right, TDLN tissue from TCF/Lef1-EGFP reporter mice counterstained for CD11c expression. GFP+CD11c+ DCs (arrows). 60x. F. GFP confocal microscopy of BRAFV600EPTEN+/−-NTC and BRAFV600EPTEN+/−-Wnt5aKD tumors in TCF/Lef1-EGFP reporter mice. NTC, non-targeted control. KD, knock-down. *P<0.05, **P<0.005, ***P<0.0005.

Figure 3. Wnt5a Conditions DCs to Drive Regulatory T-cell Differentiation. A. Flow cytometry of CD4+FoxP3+ Tregs in TDLNs of melanoma-bearing Tyr::CreER;BrafCA;Ptenlox/lox mice and LNs of non-tumor-bearing control mice. B. Flow cytometry of CD4+FoxP3+ Tregs in TDLNs derived from...
BRAF<sup>V600E</sup>PTEN<sup>+/−</sup>-NTC tumor and BRAF<sup>V600E</sup>PTEN<sup>+/−</sup>-Wnt5a<sup>KD</sup> tumor-bearing mice. C. CD4<sup>+</sup>FoxP3<sup>+</sup> Treg differentiation assay using pre-conditioned DCs. *P-values, two-way ANOVA. D. Treg differentiation assay using DCs pre-incubated with BRAF<sup>V600E</sup>PTEN<sup>+/−</sup>-NTC and BRAF<sup>V600E</sup>PTEN<sup>+/−</sup>-Wnt5a<sup>KD</sup>-derived CM. E. Treg differentiation assay using DCs purified from BRAF<sup>V600E</sup>PTEN<sup>+/−</sup>-NTC and BRAF<sup>V600E</sup>PTEN<sup>+/−</sup>-Wnt5a<sup>KD</sup> tumors. *<em>P</em>&lt;0.05, **<em>P</em>&lt;0.005, ***<em>P</em>&lt;0.0005.

Figure 4. Melanoma-derived Wnt5a Upregulates IDO Expression and Activity in DCs. A. qrt-PCR analysis of IDO expression by DCs purified from LN and tumor tissues from <em>Tyr::CreER;Braf<sup>Ca</sup>;Pten<sup>lox/lox</sup></em> mice. DC IDO levels normalized to no tumor LN DC IDO levels or to BMDC IDO levels in non-tumor-bearing mice. *<em>P</em>-value, one-way ANOVA. B. qrt-PCR analysis of IDO expression by DCs derived from BRAF<sup>V600E</sup>PTEN<sup>+/−</sup>-NTC tumor and BRAF<sup>V600E</sup>PTEN<sup>+/−</sup>-Wnt5a<sup>KD</sup> tumors. Normalized to BRAF<sup>V600E</sup>PTEN<sup>+/−</sup>-NTC tumors. C. IDO Western blot of BMDCs treated with Wnt3a or Wnt5a ± XAV939 for 24 or 48 hrs. UT, untreated. IFN<sub>γ</sub>, positive control. D. COS7 cells transfected with an IDO<sub>prom</sub>-luciferase reporter plasmid treated with Wnt3a or Wnt5a. *<em>P</em>-value, one-way ANOVA. E. HPLC analysis of kynurenine levels in CM derived from BMDCs treated with Wnt3a, Wnt5a, and IFN<sub>γ</sub>. *<em>P</em>-value, one-way ANOVA. F. ChIP analysis of Wnt3a- and Wnt5a-induced β-catenin binding to the IDO promoter in BMDCs, 24 hour treatment. IFN<sub>γ</sub> induction of STAT1 binding to the IDO promoter, positive control. *<em>P</em>-value, one-way ANOVA. G. qrt-PCR analysis of IDO expression by the DC2.4 cell line genetically silenced for β-catenin. H. IDO and β-catenin Western blot analysis of BMDCs co-incubated with B16 melanoma CM in the presence of anti-Wnt5a Ab or sFRP3. *<em>P</em>&lt;0.05, **<em>P</em>&lt;0.005, ***<em>P</em>&lt;0.0005.

Figure 5. Wnt5a Conditions DCs to Promote Regulatory T-Cell Differentiation in an IDO-dependent Manner. A. Wnt5a or Wnt3a pre-conditioned DCs were co-incubated with naïve
splenic CD4⁺CD62L⁺ T cells ± D,L-1-MT and analyzed by flow cytometry. Data are normalized to untreated (UT) DCs. P-values, one-way ANOVA. B. WT vs IDO⁻/⁻ DCs treated with vehicle control (Ctrl) vs Wnt5a were injected into the footpad of syngeneic FoxP3-mRFP mice and draining LNs were harvested for CD4⁺RFP⁺ flow cytometry. P-values, one-way ANOVA. C. BRAF^V600EPTEN⁻/-NTC and BRAF^V600EPTEN⁻/-Wnt5aKD cell lines were implanted into the chest wall of syngeneic WT mice and tumor growth was monitored. P-value, Mann-Whitney U-test. D. Axillary LNs were resected from BRAF^V600EPTEN⁻/-NTC and BRAF^V600EPTEN⁻/-Wnt5aKD tumor-bearing mice and DNA was extracted for semi-quantitative PCR analysis of the puromycin resistance gene as a surrogate marker of melanoma metastasis. P-value, Mann-Whitney U-test. E. BRAF^V600EPTEN⁻/-NTC and BRAF^V600EPTEN⁻/-Wnt5aKD tumor volume ratios were measured in syngeneic mice undergoing treatment with either twice daily D-1-MT or hydroxyamidine IDO1 inhibitor versus vehicle. *P<0.05, **P<0.005, ***P<0.0005.

Figure 6. Silencing PORCN Expression in the B16 Melanoma Model Enhances Antitumor Immunity and Suppresses Tumor Progression In vivo. A. Schematic of PORCN-mediated Wnt palmitoylation and secretion in melanoma. B. Luciferase assay of 293T-TCF/LEF1 cells incubated with CM from B16-PORCN⁰⁰/wt/B16-NTC cells. C. CM from HA-Wnt5a transfected B16-NTC cells treated with increasing concentrations of C59 (left) and HA-sRIII or HA-Wnt5a transfected B16-PORCN⁰⁰ cells (right). IP (top) and Western blot (bottom) performed using a mouse and rabbit anti-HA antibody, respectively. C59, PORCN inhibitor. HA-sRIII, HA-tagged soluble ectodomain of type III TGFβ receptor, positive control for protein secretion. D. Wnt5a detection in the CM of B16-NTC and B16-PORCN⁰⁰ cells by ELISA. E. B16-NTC and B16-PORCN⁰⁰ cells were implanted in syngeneic mice and tumor growth monitored. P-value, Mann-Whitney U-test. F. Flow
cytometry analysis of tumor-infiltrating CD3⁺CD8⁺ T lymphocytes in B16-PORCN⁺/B16-NTC tumors. *P<0.05,**P<0.005.

**Figure 7. Immunotherapeutic Implications of Wnt-β-catenin Signaling Blockade and Evaluation of β-catenin Pathway Activation in Sentinel LN-derived DCs.**

A. *left*, Wnt5a ELISA of CM harvested from B16 and BRAF⁶⁰⁰EPTEN⁻/⁻ cell lines ± C59. *right*, Wnt5a ELISA of CM harvested from WM266 cell line ± C59. B. CM harvested from B16 cells ± C59 co-incubated with BMDCs and analyzed by IDO and β-catenin Western blot. C. B16 melanoma-bearing mice were treated with C59 alone at 5 mg/kg/day (oral gavage), 100 μg anti-CTLA-4 mAb alone (intra-peritoneal injection) every 3 days, or the combination. UT, vehicle control and isotype control antibody. *P*-value, Mann-Whitney U-test. D. Dextramer flow cytometry analysis of TRP2-specific CD8⁺ T cells in B16/F10 tumors treated with anti-CTLA-4 mAb and C59. *P*-value, one-way ANOVA. E. Melanoma-derived Wnt5a induces the β-catenin signaling pathway in nearby DCs, upregulating IDO and promoting Treg differentiation in the TME. PORCN represents a novel immunotherapeutic target. F. Human sentinel LN-derived DC expression of β-catenin target genes is associated with melanoma disease burden. *top*, primary melanoma Breslow thickness and LN involvement of melanoma patients. *bottom*, lymph node disease burden based on diameters of melanoma tissue deposits in lymph node tissues. IB,IIIA,IIIC, pathologic staging. IIIC→IV, progression to stage IV disease. *P*-value, two-way ANOVA. *P<0.05,**P<0.005.

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Figure 1

A. 

B. 

C. 

D. 

E.
Figure 2

A. Relative mRNA Level

B. Relative mRNA Level

C. Tumor

D. Tumor

E. Tumor

F. GFP+ Stromal Cells
Figure 3

A. % CD4+ FoxP3+ Tregs

- No Tumor LN
- TDLN

B. % CD4+ FoxP3+ Tregs

- NTC
- Wnt5a-KD

C. Purify CD4+CD68+ cells
Wash BMDCs

D. HARVEST DCs
In vitro Treg Assay

E. Conditioned DCs with CD4+ TFC
In vitro Treg Assay

Legend:
- 1:1
- 5:1
- 10:1

UT
IFN-γ
TGF-β
LPS/IFN-α
Whit3a
Whit5a

BRAF^V600E PTEN^+/−-NTC
BRAF^V600E PTEN^+/−-Wnt5aKD

**

**

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**Figure 4**

A. Relative IDO mRNA level in different cell types.

B. Relative IDO mRNA level in BRAF<sup>V600E</sup>-PTEN<sup>+/−</sup>-NTC and BRAF<sup>V600E</sup>-PTEN<sup>+/−</sup>-Wnt5aKD.

C. Western blot analysis for IDO, β-actin, and Wnt5a expression.

D. Relative luciferase activity with different treatments.

E. Fold-increase in kynurenine with different treatments.

F. Binding to IDO promoter with different treatments.

G. Relative IDO mRNA level in NTC and β-catenin-KD.

H. Western blot analysis for IDO, β-catenin, and β-actin expression with different anti-Wnt5a antibody and sFRP3 concentrations.
Figure 6

A. Diagram of Melanoma Cell with Wnt and Porcn pathways indicated.

B. Graph showing Relative Luciferase Activity with B16-NTC and B16-PORCN\textsuperscript{KD}.

C. Western blot images of Total Lysate and anti-HA blot with B16-NTC, B16-PORCN\textsuperscript{KD}, sRIII-HA, Wnt5a-HA, and Light chain.

D. Bar graph of Corrected Abs (450 nm) with B16-NTC and B16-PORCN\textsuperscript{KD}.

E. Line graph showing Tumor Volume (cm\textsuperscript{3}) with B16-NTC and B16-PORCN\textsuperscript{KD} over days 10, 15, 20, and 25.

F. Bar graph of % CD3\textsuperscript{+}CD8\textsuperscript{+} T Cells with B16-NTC and B16-PORCN\textsuperscript{KD}.

* indicates statistical significance.
Figure 7

A. C59 (nM)

B. 0 0.1 1 10 100

C59 (nM)

IDO

β-catenin

β-actin

C.

Tumor Volume (cm³)

Day

0 0.5 1 1.5 2

0 0.5 1 1.5 2

0 0.5 1 1.5 2

0 0.5 1 1.5 2

D.

% CD8⁺K⁺ T cells

TRP2-specific T cells

UT

C59

anti-CTLA-4 mAb

C59 + anti-CTLA-4 mAb

E.

Wnt5a

Porcn

C59

Wnt5a

β-catenin

DC

Naïve CD4⁺ T cells

FoxP3⁺ Tregs

F.

Relative mRNA Level

tcf7

Ido1

ccnd1

0 1 2 3

0 1 2 3

0 1 2 3

0 1 2 3

0 1 2 3

0 1 2 3

IB IIIA IIIC IIIC → IV

Lymph Node Disease Burden (mm)
Cancer Immunology Research

Melanoma-derived Wnt5a Promotes Local Dendritic-Cell Expression of IDO and Immunotolerance: Opportunities for Pharmacologic Enhancement of Immunotherapy

Alisha Holtzhausen, Fei Zhao, Kathy Evans, et al.


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