

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

Alisha Holtzhausen¹, Fei Zhao², Kathy S. Evans², Masahito Tsutsui³, Ciriana Orabona⁴, Douglas S. Tyler³, and Brent A. Hanks²

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. *Cancer Immunol Res*; 3(9): 1082–95. ©2015 AACR.

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 popu-

lations (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 immunoregulatory 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). Although tryptophan depletion dampens T-cell proliferation, the generation of kynurenine drives the differentiation of Tregs (7). While 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 the anti-CTLA-4 and anti-PD-1 monoclonal antibodies (mAb) have demonstrated the importance of the immune system in regulating melanoma progression and further illustrates the significance of immunoregulatory 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,

¹Department of Pharmacology and Cell Biology, Duke University Medical Center, Durham, North Carolina. ²Department of Medicine, Division of Medical Oncology, Duke University Medical Center, Durham, North Carolina. ³Department of Surgery, Division of Surgical Oncology, Duke University Medical Center, Durham, North Carolina. ⁴Department of Experimental Medicine, Section of Pharmacology, University of Perugia, Perugia, Italy.

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A. Holtzhausen and F. Zhao contributed equally to this article.

Current address for D.S. Tyler: Department of Surgery, University of Texas Medical Branch, Galveston, TX 77555.

Corresponding Author: Brent A. Hanks, Duke University Medical Center, 203 Research Drive, MSRB1, Box 2639, Durham, NC 27710. Phone: 919-684-1995; Fax: 919-613-1728; E-mail: brent.hanks@duke.edu

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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 immunoregulatory enzyme or whether expression of β -catenin target genes in DCs may reflect an immunotolerant 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). Although 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.

Materials and Methods

Mice

BALB/c(H-2^d), C57BL/6(H-2^b), *Tyr::CreER;Braf^{CA};Pten^{lox/lox}*(H-2^b), Tg(TCF/Lef1-HIST1H2BB/EGFP)61Hadj/J(H-2^b), and B6.129-*Indo^{miAlm}/J*(H-2^b) mice were purchased from Jackson Labs. FoxP3-mRFP mice (H-2^b) were a gift from H.K. Lyerly (Duke University, Durham, NC). All experiments performed under an Institutional Animal Care and Use Committee-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 from L. Ring, Ludwig-Maximilians-Universität, München, Germany) 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 Eagle's minimum essential medium, 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 Duke University Medical Center

(Duke University, Durham, NC; ref. 26). The BRAF^{V600E}PTEN^{-/-} cell line was generated by spontaneous immortalization of a resected *Tyr::CreER;Braf^{CA};Pten^{lox/lox}* tumor and cultured in RPMI, 10% FBS. The DC2.4 cell line (a gift from K. Rock, University of Massachusetts, Worcester, MA) was maintained in RPMI, 10% FBS.

Stable cell line generation

B16-PORCN^{KD}/B16-NTC cell lines were generated using a *Porcn*-targeted shRNA-expressing or control shRNA-expressing lentivirus followed by puromycin selection. The BRAF^{V600E}PTEN^{-/-}-Wnt5a^{KD} and BRAF^{V600E}PTEN^{-/-}-NTC stable cell lines were generated similarly.

Murine bone marrow-derived DCs (BMDC) were generated as previously described (27) and purified using CD11c microbeads (Miltenyi Biotec) according to the manufacturer's protocol.

Human monocyte-derived DCs 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 an 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⁺ 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, and HA (HA11) from Covance. PTEN, p-ERK, t-ERK, and Stat1 were purchased from Cell Signaling Technology and β -actin (AC-15) from Sigma. The following antibodies were purchased from BD Pharmingen: I-A^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-Alexa Fluor 488 antibody were purchased from Invitrogen, Wnt5a from R&D Systems, and gp100 [EP4863(2)] from Abcam. PCNA (F-2), S100 β , TRP1 (H-90), TRP2 (D-18), and tyrosinase (H-109) antibodies were purchased from Santa Cruz Biotechnology. Wnt3a, Wnt5a, and IFN γ ligands were purchased from R&D Systems. Both the XAV939 and 1-MT inhibitors were purchased from Sigma Aldrich: D/L-1-MT for *in vitro* studies and D-1-MT for *in vivo* studies. Hydroxyamidine INCB024360 analogue (cat# 205493; ref. 29) and C59 inhibitor were purchased from MedKoo Biosciences and Cellagen Technology, respectively.

Chromatin immunoprecipitation assays

Purified DCs were treated with Wnt3a, Wnt5a, or IFN γ , and chromatin immunoprecipitation assays (ChIP) were performed using the SimpleChIP Plus Enzymatic Chromatin IP Kit (Cell Signaling Technology).

Luciferase assays

COS7 cells were transfected with the pGL2-mIDO1 firefly luciferase reporter plasmid (a gift from A. Muller, Lankenau Institute for Medical Research, Philadelphia, PA), treated with Wnt3a, Wnt5a, and IFN γ . IDO promoter activation was analyzed using the Dual-Glo Luciferase Assay System. Wnt activity in

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conditioned medium (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^{-/-}-Wnt5a^{KD}, and BRAF^{V600E}PTEN^{-/-}-NTC cell lines were pulsed with [³H]thymidine (1 μCi, Perkin Elmer) for 24 and 48 hours.

ELISA

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

Flow cytometry

Cells were stained according to the 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-labeled H-2K^b-TRP2 dextramer (Immudex).

Measurement of intracellular Ca²⁺

DC intracellular Ca²⁺ 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-2^d) were treated with Wnt3a or Wnt5a for 18 to 48 hours at 37°C, washed, and replated at a 10:1, 5:1, or 1:1 T-cell:DC ratio with purified allogeneic (H-2^b)-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-2^b) were treated with Wnt5a or vehicle control for 48 hours 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

RNA was isolated using the RNeasy Mini Kit (Qiagen) and reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad) 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 S1. Relative transcript levels were calculated according to the 2^{-ΔΔC_t} method; data were normalized to GAPDH.

PCR-based quantitation of melanoma lymph node 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 semiquantitative PCR using the Apex Hot Start 2.0 Master Mix (Genesee).

Microarray analysis

Microarray datasets publicly available on the OncoPrint Cancer Profiling Database (OncoPrint 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 × 10³), B16-NTC/B16-PORCN^{KD} (1 × 10⁵), and BRAF^{V600E}PTEN^{-/-}-Wnt5a^{KD}/BRAF^{V600E}PTEN^{-/-}-NTC (50 × 10⁵) cells were implanted into syngeneic C57BL/6 mice and tumor growth monitored by caliper measurement. Tumor and TDLN tissues were resected between days 14 and 21. Tyr::CreER;Braf^{CA};Pten^{lox/lox} transgenic mice were treated with topical 4-HT (Sigma, 20 mmol/L) for 2 days to induce primary melanoma development. C59 was administered daily by oral gavage (5 mg/kg/d), 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 nonparametric one-way ANOVA and two-way ANOVA followed by *post hoc* multicomparison testing. Wnt5a expression levels were correlated with FoxP3 expression levels based on a nonparametric 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 SEM.

Human lymph node DC isolation

Eight patients were consented and accrued onto the lymph node study approved by the DUMC Institutional Review Board. Lymphoscintigraphy identified the sentinel lymph node for harvest and biopsy. Single-cell suspensions were generated from a sample of sentinel lymph node 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

On the basis of 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. S1) and found a significant correlation in *Wnt5a* and *FoxP3* expression in human melanoma tissues (Fig. 1A).

Both preclinical and clinical data suggest that Wnt5a promotes melanoma metastasis (16, 17). On the basis of 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 cocultured the 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 coculturing 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 affecting local DC function in the human melanoma microenvironment.

Melanoma-derived Wnt5a induces β -catenin signaling pathway activation in DCs *in vitro* and *in vivo*

On the basis of 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. S2).

To study this Wnt-mediated paracrine signaling pathway *in vivo*, we utilized the *Tyr::CreER;Braf^{CA};Pten^{lox/lox}* transgenic melanoma mouse model which closely recapitulates human melanomas by expressing a melanocyte-specific *BRAF^{V600E}* activation mutation in the absence of the *PTEN* tumor suppressor (Supplementary Fig. S3A and S3B; ref. 19). To determine whether 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⁺IA^{b+} DC (TIDC) populations using quantitative RT-PCR and compared these expression levels with control BMDCs isolated from non-tumor-bearing mice (Supplementary Fig. S4A and S4B). 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 whether 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;Braf^{CA};Pten^{lox/lox}* mice and purified CD11c⁺ DCs for further analysis (Supplementary Fig. S4C). These studies showed enhanced levels of β -catenin–responsive genes in TDLN DCs relative to distant lymph node DCs and lymph node 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;Braf^{CA};Pten^{lox/lox}* mice (Supplementary Fig. S5) and implanted this *BRAF^{V600E}PTEN^{−/−}* cell line into syngeneic Tg(TCF/Lef1-HIST1H2BB/EGFP)61Hadj/J transgenic β -catenin–dependent GFP reporter mice (32). After primary

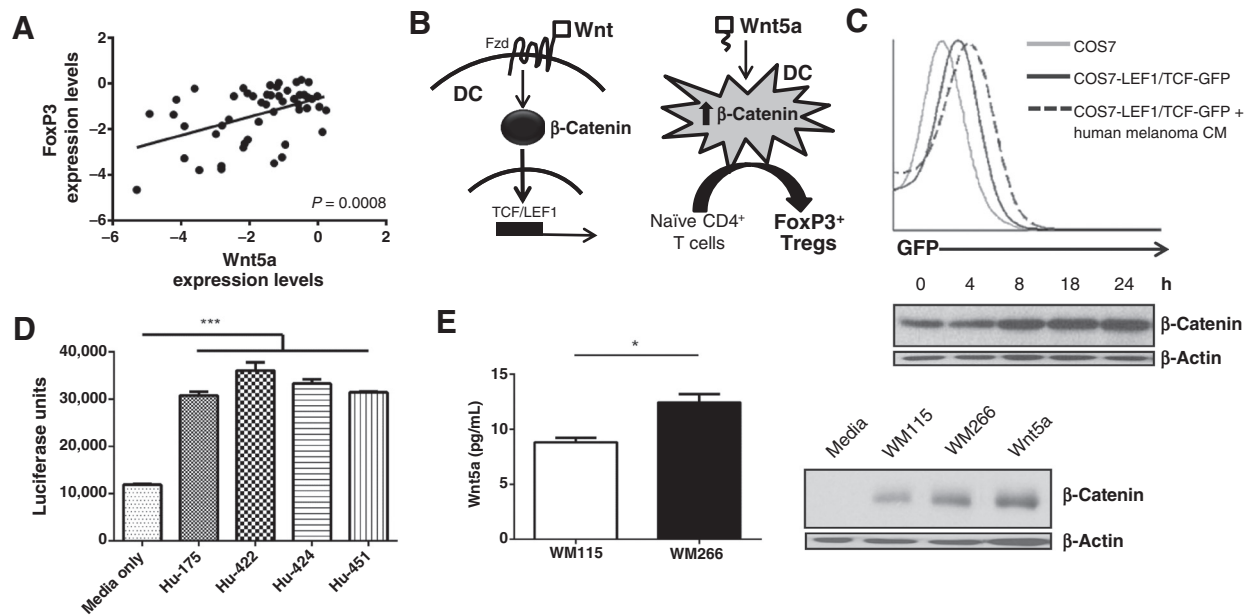
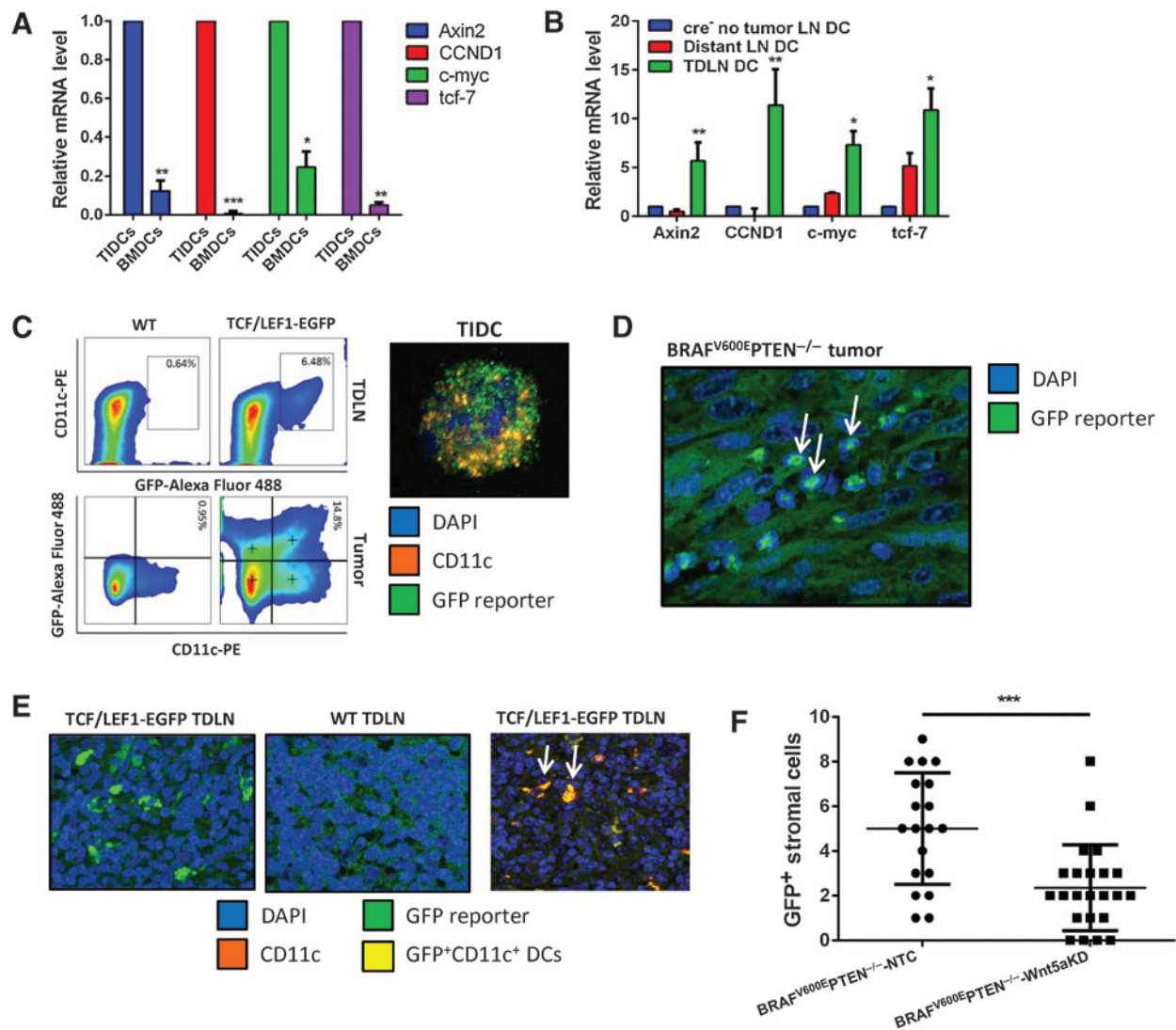


Figure 1.

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

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**Figure 2.**

Murine melanomas induce paracrine β -catenin signaling activation in tumor and TDLN DCs *in vivo*. A, quantitative RT-PCR of β -catenin target gene expression by TIDCs isolated from *Tyr::CreER;Braf^{CA};Pten^{lox/lox}* primary melanomas relative to BMDCs derived from non-tumor-bearing mice. *P*, two-way ANOVA. B, quantitative RT-PCR of β -catenin target gene expression by DCs isolated from TDLNs from melanoma-bearing *Tyr::CreER;Braf^{CA};Pten^{lox/lox}* mice compared with distant lymph nodes and lymph nodes from non-tumor-bearing *cre⁻* mice. *P* value, one-way ANOVA. C, flow cytometry analysis of GFP⁺CD11c⁺ DCs from TCF/Lef1-EGFP reporter mice (left). Confocal microscopy of a GFP⁺CD11c⁺ DC isolated from a BRAF^{V600E}PTEN^{-/-} primary melanoma (right). WT, wild type. D, GFP confocal microscopy of BRAF^{V600E}PTEN^{-/-} melanoma tissue resected from a TCF/Lef1-EGFP reporter mouse. Magnification, $\times 100$. E, GFP confocal microscopy of TDLN tissue resected from TCF/Lef1-EGFP reporter mice counterstained for CD11c expression. GFP⁺CD11c⁺ DCs (arrows). Magnification, $\times 60$. F, GFP confocal microscopy of BRAF^{V600E}PTEN^{-/-}-NTC and BRAF^{V600E}PTEN^{-/-}-Wnt5a^{KD} tumors in TCF/Lef1-EGFP reporter mice. NTC, nontargeted control. KD, knockdown. *, *P* < 0.05; **, *P* < 0.005; ***, *P* < 0.0005.

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;Braf^{CA};Pten^{lox/lox}* melanoma tumors express high levels of *Wnt5a* (Supplementary Fig. S3C and S3D). To determine the contribution of *Wnt5a* in regulating the DC β -catenin signaling pathway observed above, we genetically silenced *Wnt5a* expression in the BRAF^{V600E}

PTEN^{-/-} melanoma cell line (BRAF^{V600E}PTEN^{-/-}-Wnt5a^{KD}) 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. S7A and S7B). Using confocal microscopy, we determined BRAF^{V600E}PTEN^{-/-}-Wnt5a^{KD} 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; Brafc^{CA};Pten^{lox/lox}* mice bearing Wnt5a-expressing melanoma tumors indicate that these tissues harbor increased levels of CD4⁺FoxP3⁺Tregs relative to lymph node tissues resected from non-tumor-bearing mice (Fig. 3A). In addition, BRAF^{V600E}PTEN^{-/-}-Wnt5a^{KD} tumors were noted to be associated with reduced numbers of TDLN Tregs relative to BRAF^{V600E}PTEN^{-/-}-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 pretreated purified BMDCs with recombinant Wnt5a and several other known DC stimuli as controls. These DCs were harvested and cocultured with purified splenic CD4⁺T cells and then analyzed by flow cytometry to quantify CD4⁺FoxP3⁺Tregs (Fig. 3C and Supplementary Fig. S8). In contrast to other stimuli, preconditioning 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 cocultured DCs with CM harvested from the BRAF^{V600E}PTEN^{-/-}-Wnt5a^{KD} and BRAF^{V600E}PTEN^{-/-}-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^{V600E}PTEN^{-/-}-Wnt5a^{KD} and BRAF^{V600E}PTEN^{-/-}-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 IDO 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. S9A). Further ELISA studies also did not show Wnt5a to induce DC expression of IL10 or TGF β at various time points (Supplementary Fig. S9B). However, noting that other investigators have shown the *IDO1* promoter to comprise LEF1-binding elements (33), we purified TIDCs and TDLN-derived DCs from *Tyr::CreER;Brafc^{CA};Pten^{lox/lox}* transgenic mice and found these DCs to express elevated levels of IDO relative to lymph node-derived DCs purified from non-tumor-bearing hosts (Fig. 4A). To determine whether Wnt5a contributed to the differences in DC IDO expression observed in these experiments, DCs were isolated from BRAF^{V600E}PTEN^{-/-}-Wnt5a^{KD} and BRAF^{V600E}PTEN^{-/-}-NTC control tumors and analyzed for IDO expression. Indeed, these assays revealed diminished IDO expression in DCs harvested from the BRAF^{V600E}PTEN^{-/-}-Wnt5a^{KD} 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 analysis (Fig. 4C). These experiments showed lithium

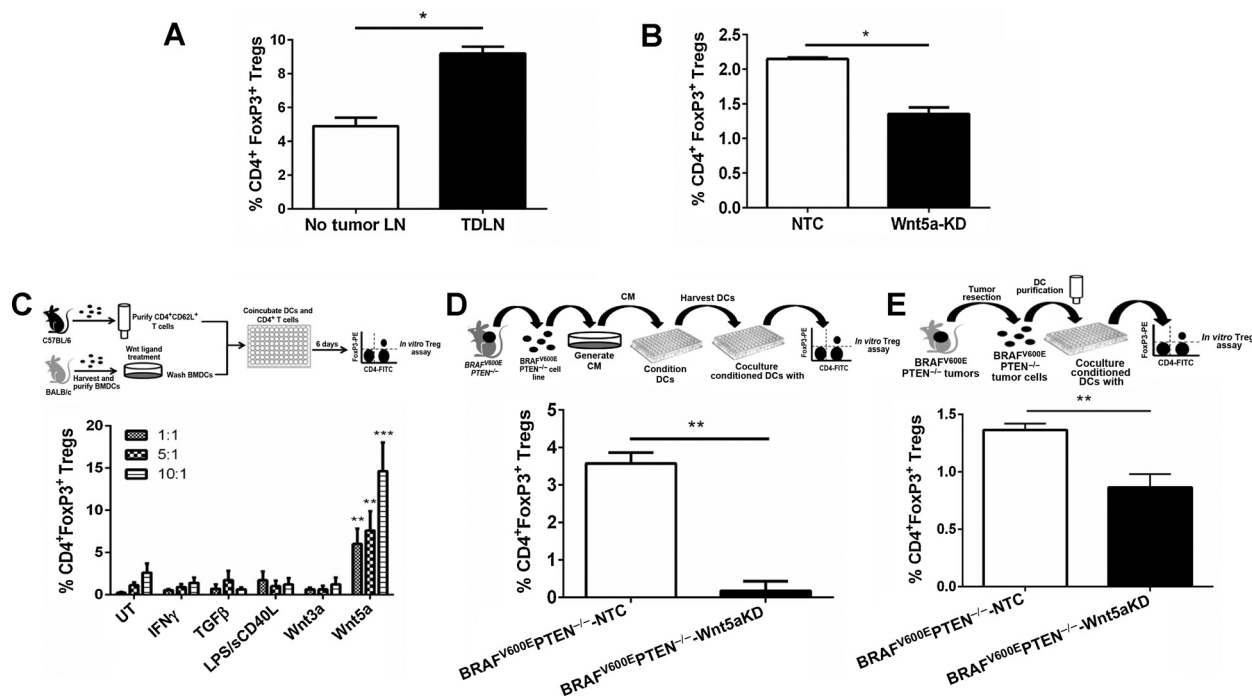
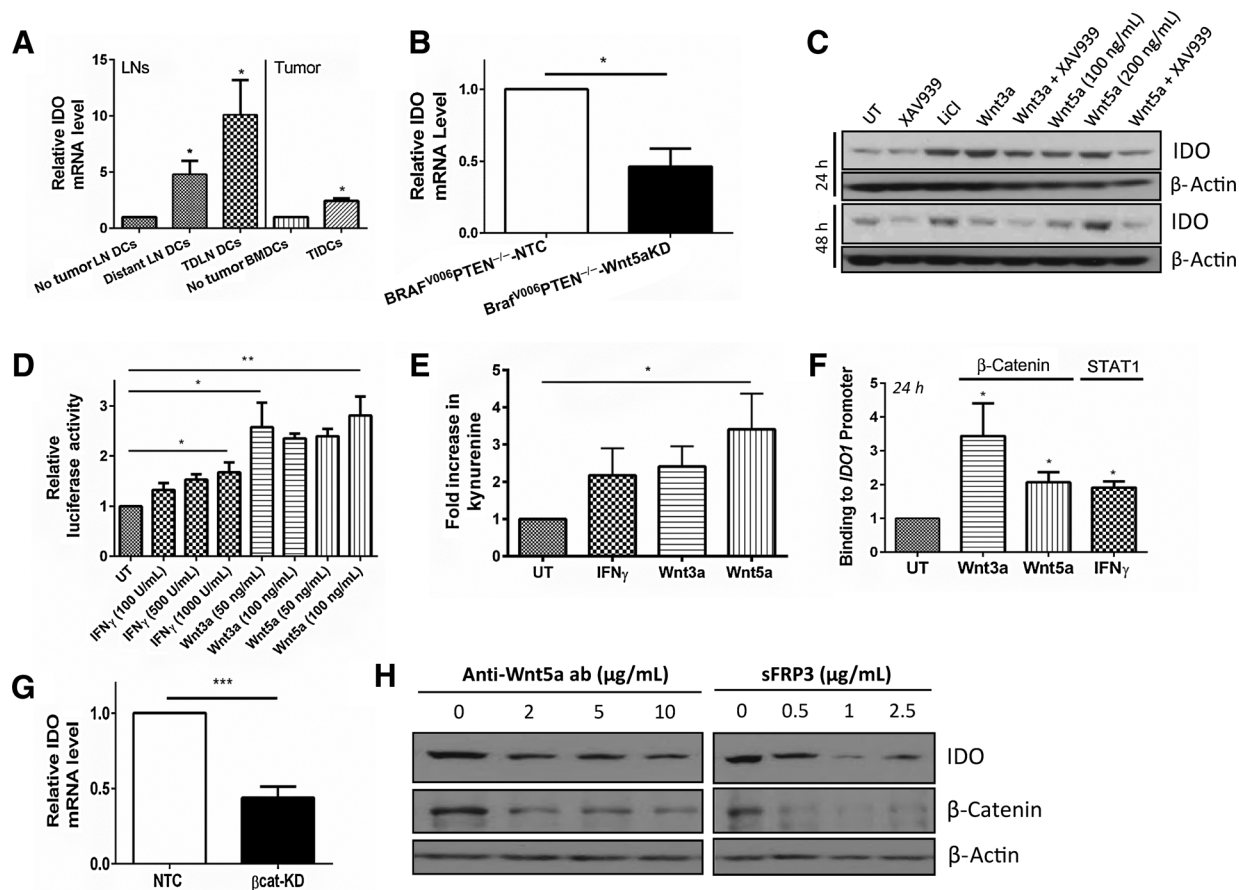


Figure 3.

Wnt5a conditions DCs to drive regulatory T-cell differentiation. A, flow cytometry of CD4⁺FoxP3⁺Tregs in TDLNs of melanoma-bearing *Tyr::CreER;Brafc^{CA};Pten^{lox/lox}* mice and LNs of non-tumor-bearing control mice. B, flow cytometry of CD4⁺FoxP3⁺Tregs in TDLNs derived from BRAF^{V600E}PTEN^{-/-}-NTC tumor and BRAF^{V600E}PTEN^{-/-}-Wnt5a^{KD} tumor-bearing mice. C, CD4⁺FoxP3⁺Treg differentiation assay using preconditioned DCs. *P*, two-way ANOVA. D, Treg differentiation assay using DCs preincubated with BRAF^{V600E}PTEN^{-/-}-NTC and BRAF^{V600E}PTEN^{-/-}-Wnt5a^{KD}-derived CM. E, Treg differentiation assay using DCs purified from BRAF^{V600E}PTEN^{-/-}-NTC and BRAF^{V600E}PTEN^{-/-}-Wnt5a^{KD} tumors. *, *P* < 0.05; **, *P* < 0.005; ***, *P* < 0.0005.

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**Figure 4.**

Melanoma-derived Wnt5a upregulates IDO expression and activity in DCs. A, quantitative RT-PCR analysis of IDO expression by DCs purified from lymph node and tumor tissues from *Tyr::CreER;Braf^{CA};Pten^{lox/lox}* mice. DC IDO levels normalized to no tumor lymph node DC IDO levels or to BMDC IDO levels in non-tumor-bearing mice. *P*, one-way ANOVA. B, quantitative RT-PCR analysis of IDO expression by DCs derived from BRAF^{V600E}PTEN^{-/-}-NTC tumor and BRAF^{V600E}PTEN^{-/-}-Wnt5a^{KD} tumors. Normalized to BRAF^{V600E}PTEN^{-/-}-NTC tumors. C, IDO Western blot analysis of BMDCs treated with Wnt3a or Wnt5a ± 1 μmol/L XAV939 for 24 or 48 hours. UT, untreated. LiCl (5 mmol/L) positive control. D, COS7 cells transfected with an IDO_{prom}-luciferase reporter plasmid treated with Wnt3a or Wnt5a. *P*, one-way ANOVA IFN γ , positive control. E, HPLC analysis of kynurenine levels in CM derived from BMDCs treated with Wnt3a, Wnt5a, and IFN γ . *P*, one-way ANOVA. F, ChIP analysis of Wnt3a- and Wnt5a-induced β -catenin binding to the IDO promoter in BMDCs, 24-hour treatment. IFN γ induction of STAT1 binding to the IDO promoter, positive control. *P*, one-way ANOVA. G, quantitative RT-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 coincubated with B16 melanoma CM in the presence of anti-Wnt5a Ab or sFRP3. *, *P* < 0.05; **, *P* < 0.005; ***, *P* < 0.0005.

chloride, a GSK-3 β 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 IFN γ 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 IFN γ (Fig. 4D). Because other investigators have previously shown that IDO expression and enzymatic activity may be differentially regulated (31, 35), we investigated whether 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 and Supplementary Fig. S10A).

Consistent with previous data, our findings suggest that the β -catenin transcription factor is important for regulating the DC tolerization program. Although previous studies have shown that

Wnt5a primarily signals via a β -catenin-independent noncanonical 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 quantitative RT-PCR expression analysis of the known Wnt ligand receptors/coreceptors in DCs demonstrates a profile that is compatible with Wnt5a-mediated activation of the β -catenin pathway (Supplementary Fig. S10B and S10C). In addition, our studies did not show any evidence that Wnt5a induces the noncanonical Ca²⁺-dependent signaling pathway in DCs (Supplementary Fig. S10D). 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

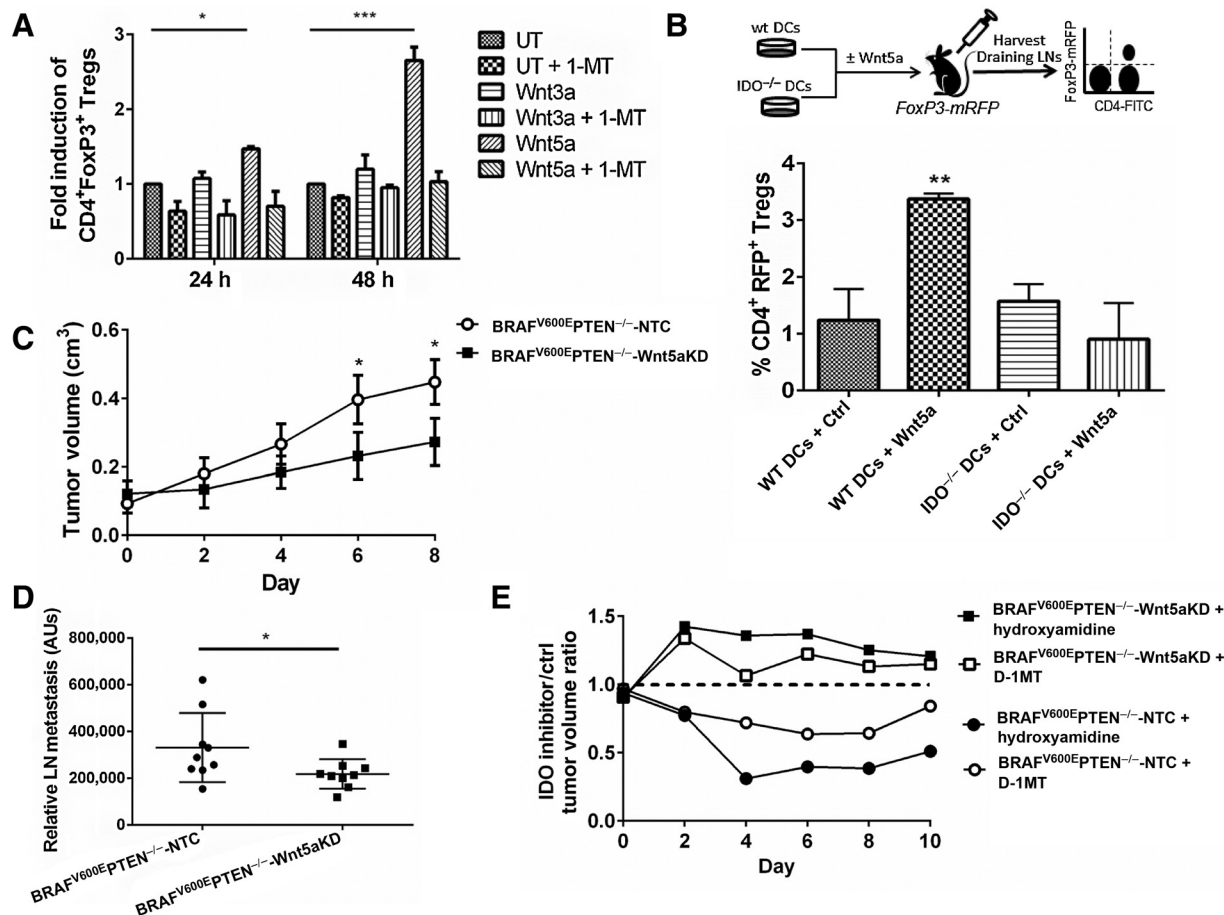


Figure 5.

Wnt5a conditions DCs to promote regulatory T-cell differentiation in an IDO-dependent manner. A, Wnt5a or Wnt3a preconditioned DCs were coincubated with naïve splenic CD4⁺CD62L⁺ T cells \pm 200 μ mol/L D,L-1-MT and analyzed by flow cytometry. Data are normalized to untreated (UT) DCs. *P*, one-way ANOVA. B, WT versus IDO^{-/-} DCs treated with vehicle control (Ctrl) versus Wnt5a were injected into the footpad of syngeneic FoxP3-mRFP mice and draining lymph nodes were harvested for CD4⁺RFP⁺ flow cytometry. *P*, one-way ANOVA. C, BRAF^{V600E}PTEN^{-/-}-NTC and BRAF^{V600E}PTEN^{-/-}-Wnt5aKD cell lines were implanted into the chest wall of syngeneic WT mice and tumor growth was monitored. *P*, Mann-Whitney *U* test. D, axillary lymph nodes were resected from BRAF^{V600E}PTEN^{-/-}-NTC and BRAF^{V600E}PTEN^{-/-}-Wnt5aKD tumor-bearing mice and DNA was extracted for semiquantitative PCR analysis of the puromycin resistance gene as a surrogate marker of melanoma metastasis. *P*, Mann-Whitney *U* test. E, BRAF^{V600E}PTEN^{-/-}-NTC and BRAF^{V600E}PTEN^{-/-}-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.

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 genetically silenced for β -catenin (DC2.4- β cat^{KD}) and BMDCs isolated from TCF/LEF1-EGFP reporter mice (Fig. 4G and Supplementary Fig. S10E). Finally, we demonstrate Wnt5a to induce IDO expression in splenic plasmacytoid DCs, suggesting that this is not a myeloid-specific phenomenon (Supplementary Fig. S10F). Together, these findings implicate β -catenin as playing an important role in the Wnt5a-mediated upregulation of IDO expression in DCs.

To confirm that melanoma-derived Wnt5a stimulates DC IDO expression, we preincubated B16 melanoma cells with anti-Wnt5a antibody prior to coculturing 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 coincubation with primary BMDCs. However,

neither anti-Wnt3a antibody nor sFRP2 affected BMDC expression of IDO in this system (Supplementary Fig. S11A and S11B). As 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. S11C). 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 IFN γ expression (42). These data raised the possibility that Wnt3a- and/or Wnt5a-dependent induction of

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IDO expression in DCs was partially IFN γ dependent. By repeating these *in vitro* stimulation assays in the presence of an anti-IFN γ blocking antibody, we determined that Wnt3a-mediated but not Wnt5a-mediated upregulation of IDO expression was dependent upon IFN γ (Supplementary Fig. S12A). These findings are further supported by quantitative RT-PCR analysis revealing that Wnt3a, but not Wnt5a, induced the expression of IFN γ by BMDCs (Supplementary Fig. S12B).

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*

On the basis of 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 naive 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^{V600E}PTEN^{-/-}Wnt5a^{KD} tumors relative to BRAF^{V600E}PTEN^{-/-}NTC tumor controls, suggesting that DC-expressed IDO also played a role in Wnt5a-mediated Treg generation *in vivo* (Figs. 3B and 4B). To confirm this hypothesis, we delivered untreated and Wnt5a-treated wild-type and

IDO1^{-/-} DCs into the footpad of FoxP3-mRFP transgenic mice and examined draining lymph node tissues for CD4⁺mRFP⁺ Tregs by flow cytometry (Supplementary Fig. S13). Although Wnt5a-stimulated wild-type DCs reproducibly enhanced Treg generation *in situ*, this effect was eliminated when Wnt5a-stimulated IDO1^{-/-} 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^{V600E}PTEN^{-/-}NTC and BRAF^{V600E}PTEN^{-/-}Wnt5a^{KD} melanoma model system, we determined that the genetic silencing of Wnt5a expression suppresses primary melanoma development and metastasis to draining lymph nodes (Fig. 5C and D and Supplementary Fig. S14). We then repeated these studies in the presence or absence of either D-1-MT or a hydroxyamide selective IDO1 inhibitor and found that inhibition of IDO activity suppressed BRAF^{V600E}PTEN^{-/-} melanoma development only in the presence of Wnt5a, suggesting that the protumorigenic properties of Wnt5a are dependent upon IDO enzymatic activity (Fig. 5E). Overall, these results indicate that IDO is critical for the immunosuppressive properties of Wnt5a in melanoma.

The PORCN acyltransferase 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). On the basis of 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

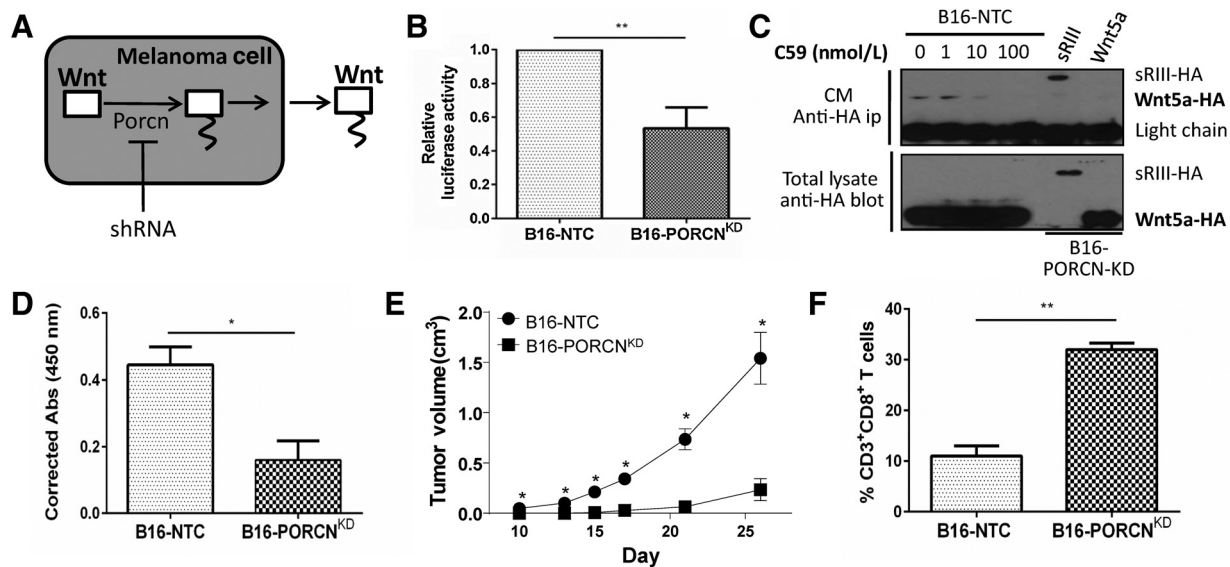


Figure 6.

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-PORCN^{KD}/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-PORCN^{KD} cells (right). Immunoprecipitation (ip; top) and Western blot analysis (bottom) performed using a mouse and rabbit anti-HA antibody, respectively. C59, PORCN inhibitor. HA-sRIII, HA-tagged soluble ectodomain of type III TGF β receptor, positive control for protein secretion. D, Wnt5a detection in the CM of B16-NTC and B16-PORCN^{KD} cells by ELISA. E, B16-NTC and B16-PORCN^{KD} cells were implanted in syngeneic mice and tumor growth monitored. P, Mann-Whitney U test. F, flow cytometry analysis of tumor-infiltrating CD3⁺CD8⁺ T lymphocytes in B16-PORCN^{KD}/B16-NTC tumors. *, $P < 0.05$; **, $P < 0.005$.

line genetically silenced for PORCN expression (B16-PORCN-KD) and a nontargeted control B16 cell line (B16-NTC; Supplementary Fig. S15A and S15B). After verifying silenced PORCN expression by quantitative RT-PCR and Western blot analysis, we cocultured 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 cocultured 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 whether the inhibition of Wnt secretion by the B16-PORCN-KD cell line affected cell proliferation or apoptosis; however, we were unable to appreciate any significant alterations in these cellular processes (Supplementary Fig. S15C–S15E). 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 BRAF^{V600E}PTEN^{-/-} tumor model in which the genetic silencing of Wnt5a expression is also associated with increased numbers of infiltrating CD8⁺ T cells (Supplementary Fig. S15F). Altogether, these data suggest that PORCN-mediated Wnt5a secretion by melanomas generates an immunotolerant TME that ultimately allows for tumor progression.

C59 inhibition of the PORCN–Wnt– β -catenin signaling pathway synergizes with anti–CTLA-4 antibody immunotherapy in the B16 melanoma model

On the basis of 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 cocultured 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 (Fig. 7A and Supplementary Fig. S16). To further determine whether the C59 inhibitor is capable of modulating IDO expression by BMDCs *in vitro*, we harvested and cocultured 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. As 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 analysis (Supplementary Fig. S17A). 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 intraperitoneal injection every 3 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 K^b-TRP2 dextramer analysis (Fig. 7D and Supplementary Fig. S17B). 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 lymph node DC expression of IDO and β -catenin target genes is associated with melanoma disease burden

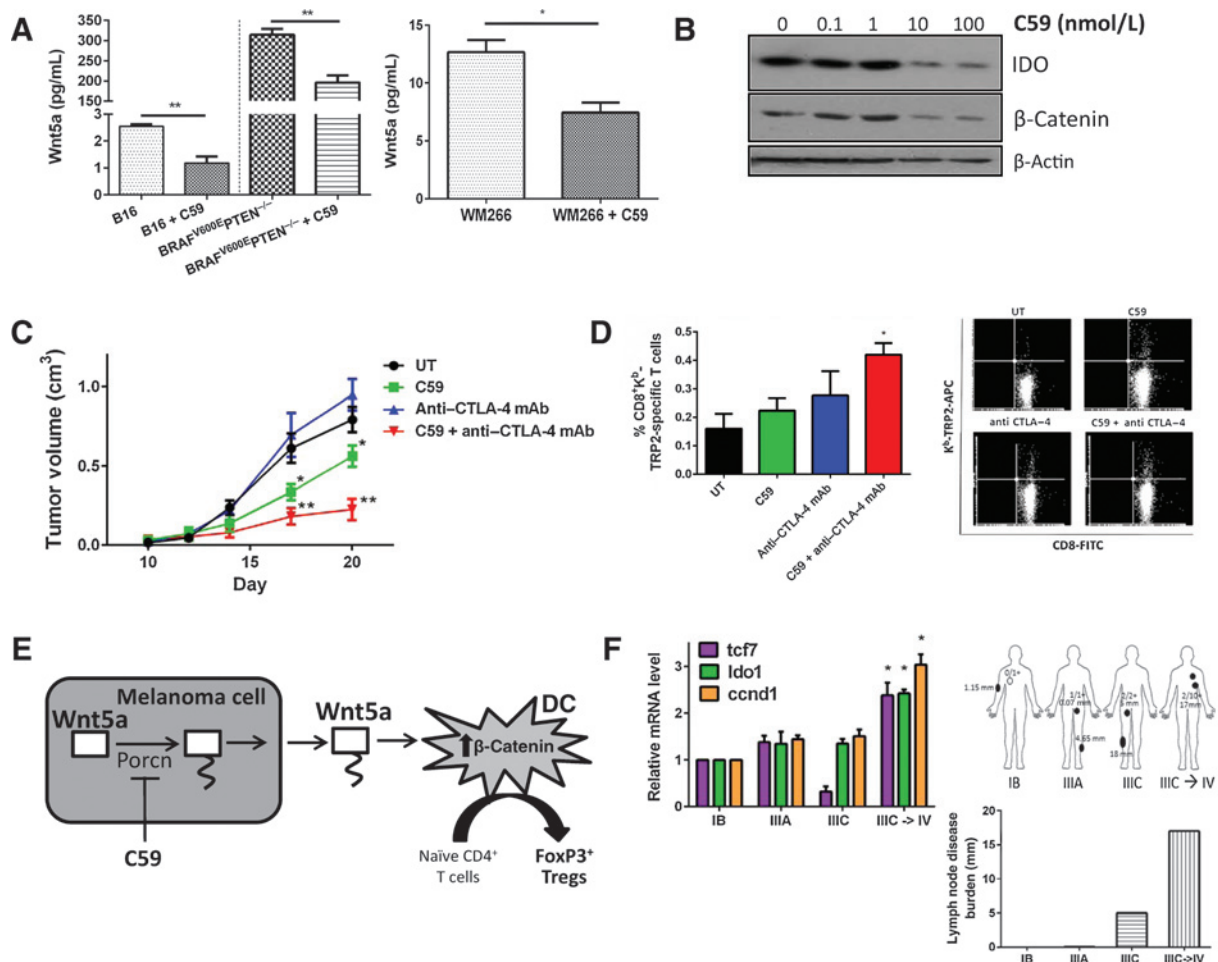
The data presented above suggest that Wnt5a induction of the β -catenin pathway promotes DC tolerization. This implies that a melanoma microenvironment comprising DC populations that exhibit a gene expression signature indicative of β -catenin signaling pathway activation is more likely to be associated with a higher tumor burden and potentially diminished relapse-free survival. We have initiated a program to investigate sentinel lymph node DC gene expression signatures as possible biomarkers for melanoma behavior and treatment response. In the initial panel of patients undergoing a staging sentinel lymph node biopsy, we isolated both myeloid and plasmacytoid DC populations and purified total RNA from these cells for downstream quantitative RT-PCR analysis (Supplementary Fig. S18A). Using this approach, we have identified a relationship between melanoma disease burden within the draining lymph node bed and elevated levels of DC *TCF7*, *IDO1*, and *CCND1* expression (Fig. 7F and Supplementary Fig. S18B). Although these data require confirmation in a larger cohort of patients, it is interesting that the only patient with metastatic progression exhibited the most significant elevations in sentinel lymph node DC-specific expression of these β -catenin-dependent genes. These findings support our previous data and indicate that lymph node DC populations may be a useful resource for biomarker development in tumor immunotherapy.

Discussion

As emerging data substantiated the role of the DCs 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 β -catenin–IDO signaling pathway that is upregulated within DC populations that reside within the TME.

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**Figure 7.**

Immunotherapeutic implications of Wnt- β -catenin signaling blockade and evaluation of β -catenin pathway activation in sentinel lymph node-derived DCs. A, Wnt5a ELISA of CM harvested from B16 and BRAF^{V600E}PTEN^{-/-} cell lines \pm 10 nmol/L C59 (left). Wnt5a ELISA of CM harvested from WM266 cell line \pm C59 (right). B, CM harvested from B16 cells \pm C59 coincubated with BMDCs and analyzed by IDO and β -catenin Western blot analysis. C, B16 melanoma-bearing mice were treated with C59 alone at 5 mg/kg/d (oral gavage), 100 μ g anti-CTLA-4 mAb alone (intraperitoneal injection) every 3 days, or the combination. UT, vehicle control and isotype control antibody. *P*, 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*, 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 lymph node-derived DC expression of β -catenin target genes is associated with melanoma disease burden. Primary melanoma Breslow thickness and lymph node involvement of melanoma patients (top). Lymph node disease burden based on diameters of melanoma tissue deposits in lymph node tissues (bottom). IB, IIIA, and IIIC, pathologic staging; IIIC \rightarrow IV, progression to stage IV disease. *P*, two-way ANOVA. *, *P* < 0.05; **, *P* < 0.005.

Although our data suggest that the Wnt3a ligand is capable of inducing signals on a transient time scale and is IFN γ dependent, the Wnt5a-induced signal is more durable and is independent of IFN γ signaling. Interestingly, these findings are similar to those reported for the differential signaling kinetics between IFN γ - and TGF β -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. Although 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 due 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 β receptor serine/threonine kinase inhibitor, another agent capable of modulating DC function, with anti-CTLA-4 antibody therapy in the *Tyr::CreER;Braf^{CA};Pten^{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, TGF β , IL10, and VEGF while augmenting TGF β 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 noncanonical pathway in DCs. The reasons for these discrepancies may include the use of supraphysiologic 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 immunotolerance. 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 IFN γ -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 antibody 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, preclinical 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; refs. 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 lymph node DCs and melanoma disease burden and limited progression-free survival. Although 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 lymph node 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 lymph node-derived DCs may offer unique insight into the immune microenvironment while also serving as a potential resource for biomarker development in tumor immunotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: A. Holtzhausen, B.A. Hanks

Development of methodology: A. Holtzhausen, B.A. Hanks

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Holtzhausen, F. Zhao, M. Tsutsui, C. Orabona, D.S. Tyler, B.A. Hanks

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Holtzhausen, F. Zhao, B.A. Hanks

Writing, review, and/or revision of the manuscript: A. Holtzhausen, F. Zhao, M. Tsutsui, D.S. Tyler, B.A. Hanks

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Holtzhausen, K. Evans, B.A. Hanks

Study supervision: B.A. Hanks

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

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