Deletion of LRPS5 and LRP6 in dendritic cells enhances antitumor immunity

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ABSTRACT
The tumor microenvironment (TME) contains high levels of the Wnt family of ligands, and aberrant Wnt-signaling occurs in many tumors. Past studies have been directed toward how the Wnt signaling cascade regulates cancer development, progression and metastasis. However, its effects on host antitumor immunity remain unknown. In this report, we show that Wnts in the TME condition dendritic cells (DCs) to a regulatory state and suppress host antitumor immunity. DC-specific deletion of Wnt co-receptors low-density lipoprotein receptor-related protein 5 and 6 (LRP5/6) in mice markedly delayed tumor growth and enhanced host antitumor immunity. Mechanistically, loss of LRP5/6-mediated signaling in DCs resulted in enhanced effector T cell differentiation and decreased regulatory T cell differentiation. This was due to increased production of pro-inflammatory cytokines and decreased production of IL-10, TGF-β1 and retinoic acid (RA). Likewise, pharmacological inhibition of the Wnts’ interaction with its cognate co-receptors LRP5/6 and Frizzled (Fzd) receptors had similar effects on tumor growth and effector T cell responses. Moreover, blocking Wnt-signaling in DCs resulted in enhanced capture of tumor-associated antigens and increased expression of immune regulatory factors such as IL-10, TGF-β1 and RA. Thus, these observations led us to hypothesize that increased Wnts in the TME could modulate effector functions of DCs to program them to a regulatory state, which effectively suppresses host effector T cell responses and induce tumor immune tolerance.

In the present study, we show that loss of co-receptors LRPS5 and LRP6 in DCs results in reduced tumor growth with enhanced antitumor immune responses. Thus, deletion of LRP5 and LRP6 in DCs resulted in decreased expression of immune regulatory factors such as IL-10, TGF-β1 and RA with concomitant increase in inflammatory cytokine production. Interestingly, loss of LRP5/6 in DCs promoted efficient capture of tumor-associated antigens by DCs and cross-priming of effector CD8+ T cells, indicating a critical role for Wnt signaling in suppressing host antitumor immunity. Furthermore, our study demonstrates that pharmacological blocking of Wnt-signaling is an attractive means of attenuating tumor-induced immune tolerance via inflammation and restores homeostasis. Furthermore, other studies have shown that ex vivo conditioning of DCs with Wnts induces regulatory T cell differentiation and suppresses effector T cell differentiation. Thus, these observations led us to hypothesize that increased Wnts in the TME could modulate effector functions of DCs to program them to a regulatory state, which effectively suppresses host effector T cell responses and induce tumor immune tolerance.

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enhancement of effector T cell responses with concomitant suppression of regulatory T cell differentiation.

**Results**

**Deletion of LRP5 and LRP6 in dendritic cells impairs tumor growth**

To determine the role of Wnts in regulating host antitumor immunity, we first analyzed the expression of different Wnt ligands in TME and tumor-draining lymph nodes (TDLNs), using B16 melanoma cells expressing ovalbumin (B16-OVA) implanted subcutaneously (s.c) in syngenic C57BL/B6 (B6) mice. As shown in Fig. 1A, we noted increased expression of multiple Wnts in the TDLN compared to the control lymph nodes (CLN). Similarly, implantation of EL4 thymoma cells expressing ovalbumin (EL4-OVA) or Lewis lung carcinoma (LLC) cells resulted in a significant increase in Wnts in the TDLN (Fig. 1B, C). Moreover, we also observed a similar pattern of Wnt expression in B16-OVA, EL4-OVA and LLC tumors isolated from tumor-bearing mice (data not shown). Our recent study has shown that DCs express both the co-receptors LRP5 and LRP6 that are critical for efficient Wnt-mediated signaling. Therefore, we reasoned that the Wnts in TME could activate canonical Wnt pathway in DCs and suppress antitumor immunity with increased tumor progression. As shown in Fig. 1D, B16-OVA tumor growth was significantly reduced in the mice lacking LRP5/6 in DCs (LRP5/6ΔDC mice) compared to control-flanked (WT) mice. Similarly, EL4-OVA or LLC tumor growth is markedly reduced in the LP5/6ΔDC mice compared to control-flanked (WT) mice (Fig. 1E, F). Taken together, these data indicated that Wnts in the TME could promote tumor progression partly through activation of Wnt-LRP5/6 signaling in DCs.

**Wnt-LRP5/6-mediated signaling in DCs suppresses antitumor immunity**

Next, we examined the effector phenotype of tumor-infiltrating lymphocytes (TILs) isolated from LRP5/6ΔDC mice. Analysis of T cells from B16 tumors and TDLN of LRP5/6ΔDC mice showed a significant increase in frequencies of IFNγ+ CD4+ , IFNγ+ CD8+ , Granzyme B+ CD8+ (GzmB+ CD8+) and TNF-α+ CD8+ T cells compared to control mice (Fig. 2A, B; S1A). Interestingly, there was a significant decrease in the frequencies of Foxp3+ CD4+ T cells, IL-10+ CD4+ Tr1 cells and IL-10+ CD8+ regulatory T cells in B16 tumor bearing LRP5/6ΔDC mice compared to the control group (Fig. 2C, D). We observed identical trends in LRP5/6ΔDC mice bearing EL4 and LLC tumors (data not shown). Next, we analyzed tumor specific immune responses by co-culturing TDLN lymphocytes and DCs pulsed with a B16 tumor extract. As shown in Fig. 2E, TDLN lymphocytes from LRP5/6ΔDC mice produced significantly higher levels of IFNγ and TNF-α and lower levels of IL-10 compared to TDLN lymphocytes from WT mice. Collectively, these results suggest that Wnt-LRPs/6 signaling in DCs promotes tumor immune tolerance through differential regulation of effector and regulatory T cell responses.

**Wnt-LRP5/6-mediated signaling in DCs suppresses effector T cell differentiation but promotes regulatory T cell differentiation**

Functional activation of T cells by DCs usually occurs in the TDLNs. Tumors condition DCs to acquire regulatory properties that mostly induce regulatory T cell responses. To
determine whether canonical Wnt signaling limits tumor antigen-specific CD8+ T cell responses, we transferred naive OT-I T cells into control WT and LRP5/6ΔDC mice bearing B16-OVA tumors. As shown in Fig. 3A–B, we noted a significant increase in the frequencies of IFNγ+, TNF-α+ and GzmB+ CD8+OT-IThy1.1+ T cells in B16-OVA tumors isolated from LRP5/6ΔDC mice compared to control mice. These results suggest that activation of Wnt-LRP5/6-mediated signaling in DCs suppresses the differentiation of effector CD8+ T cells in response to tumor. Past studies have shown

![Figure 2](image2.png)

Figure 2. Wnt ligands in the TME activate the β-catenin/TCF pathway in DCs and DC-specific deletion of LRP5/6 enhances antitumor immunity in mice. (A–D) Representative dot plots and percentages of IFNγ+CD4+, IFNγ+CD8+, Granzyme B+CD8+, TNF-α+CD8+, IL-10+CD4+, Foxp3+CD4+ and IL-10+CD8+ T cells isolated from MO4 tumors in Control-FL and LRP5/6ΔDC mice on day 14 post-inoculation (n = 6). (E) Cytokine concentrations in supernatants obtained after culture of TDLN lymphocytes with WT DCs loaded with B16 lysate for 48 h (n = 5). Data represents one of two experiments with similar results and shows mean values ±SEM. *p < 0.05; **p < 0.01; ***p < 0.001.

![Figure 3](image3.png)

Figure 3. Loss of LRPS/6 in DCs promotes effector T cell differentiation whereas limits regulatory T cell differentiation. (A, B) In vivo OT-I CD8+ T cell differentiation in Control-FL and LRP5/6ΔDC mice in response to B16-OVA (MO4) tumors. Representative dot plots and percentages percentage of OVA-specific IFNγ+CD8+, Granzyme B+CD8+ and TNF-α+CD8+ T cells in tumors isolated from Control-FL and LRP5/6ΔDC mice (n = 6). (C, D) In vivo OT-II Treg differentiation in Control-FL and LRP5/6ΔDC mice in response to B16-OVA (MO4) tumors (n = 6). (E) MO4 melanoma and (F) EG7 tumor growth in LRP5/6ΔDC in response to α-CD4 antibody or α-CD8 antibody treatment (n = 6). Data represents cumulative percentage of OVA-specific Foxp3+CD4+ T cells in TDLNs and tumors isolated from Control-FL and LRP5/6ΔDC mice (n = 5). *p < 0.05; **p < 0.01; ***p < 0.001.
that activation of β-catenin in DCs promotes regulatory T cell responses while suppressing effector T cell responses.13,18,19 Therefore, we hypothesized that tumor-induced activation of the Wnt-LRP5/6 pathway imparts a regulatory phenotype on DCs that promotes regulatory T cell responses. To test this hypothesis, LRP5/6-ACD and control mice bearing B16-OVA tumors were adoptively transferred with naive OT-II Thy1.1+ T cells on day 9 post tumor induction as described in our previous study.20 As shown in Fig. 3C–D, tumor bearing LRP5/6-ACD mice showed a significant reduction in OVA-specific induced Foxp3+ CD4+ Treg cells in TDLN and tumors compared to control mice. Based on these observations, we hypothesize that the reduced tumor growth in LRP5/6-ACD mice is due to enhanced effector T cell responses and reduced regulatory T cell responses. To test this, we depleted CD4+ or CD8+ T cells in LRP5/6-ACD mice using antibodies against CD4+ or CD8+ cells on the same day as B16-OVA (MO4) or EL4-OVA (EG7) tumor implantation and again on day 15 after tumor implantation. α-CD8+ or α-CD4+ treatment resulted in a significant increase in tumor size compared to untreated mice in LRP5/6-ACD mice (Fig. 3E, F), indicating that CD4+ and CD8+ cells are at least in part responsible for delayed tumor growth in mice lacking canonical Wnt signaling in DCs. Taken together, these data demonstrate that the activation of canonical Wnt signaling in DCs differentially regulates effector and regulatory T cell differentiation, and induces active immune tolerance to tumors.

**Canonical Wnt signaling in DCs induces the expression of immune regulatory factors while suppressing the expression of inflammatory cytokines**

DCs control the fate of naive T cell differentiation through the secretion of various inflammatory or anti-inflammatory cytokines.8,24 The above results prompted us to assess whether canonical Wnt signaling regulates DC function through the expression of inflammatory and anti-inflammatory cytokines in response to tumors. Accordingly, DCs isolated from the TDLN of LRP5/6-ACD mice bearing B16 tumors showed a significant reduction in mRNA levels for immune regulatory genes such as IL-10, TGF-β1, and Aldh1a2 in LRP5/6-ACD DCs compared to Control-FL DCs (Fig. 4A). In contrast, TDLN DCs from LRP5/6-ACD mice showed significant increases in the expression of inflammatory cytokines such as IL-6, TNF-α, IL-12p19 and IL-12p35 compared to the TDLN DCs from Control-FL mice (Fig. 4B). Our previous studies have shown that induction of IL-10, TGF-β1, and Aldh1a2 in DCs is dependent on β-catenin pathway.13,14,19,20 So, we analyzed whether lack of LRP5/6 in DCs during tumor progression affects activation of downstream signaling pathway, particularly activation of β-catenin/TCF pathway. As shown in Fig. 4C–D, deletion of LRP5/6 in DCs resulted in a marked decrease in β-catenin activation and expression of its target gene Axin2. Consistent with previous observations,15,25,26 Wnts such as 3a, 5b and 16 that are present in the TME also activates β-catenin/TCF pathway in DCs (Fig. 4E). Altogether, these data demonstrate that the canonical Wnt-signaling pathway in DCs promotes Treg cell

**Figure 4.** Loss of LRP5/6 in DCs differentially regulates pro- and anti-inflammatory molecules. (A–B) Quantitative real-time PCR analysis of Il-12p40, Il-12p35, Il-6, Il-12p19, Il10, Aldh1a1 and Aldh1a2 mRNA expression in CD11c+ DCs isolated from the TDLN of WTFL/FL and LRP5/6-ACD mice on day 9 post-B16 tumor inoculation (n = 3); (C) Representative histograms of active β-catenin expression in CD11c+ cells from MO4 tumor TDLN of LRP5/6-ACD and Control-FL mice on day 9 after tumor inoculation. (D) Axin2 mRNA expression analyzed by qRT-PCR in TDLNs and CLNs of LRP5/6-ACD and Control-FL mice on day 9 after inoculation (n = 4). The result represents fold increase over the CLNs. (E) Representative histograms showing β-gal expression in CD11c+ cells from TCF/LEF-LacZ reporter mice after treatment with or without Wnt2b, Wnt3a, Wnt5a, Wnt5b, or Wnt16b (500 ng/mL) for 24 h in vitro. DCs were pooled from five to six mice, and the experiment was repeated at least two times. Data represents one of two experiments with similar results. *p < 0.05; **p < 0.01; ***p < 0.001.
differentiation by inducing immune regulatory factors but suppresses effector T cell differentiation by limiting the expression of inflammatory cytokines.

**Deletion of LRP5/6 in DCs augments antigen uptake and cross-priming of CD8+ T cells**

Effective priming of T cells by DCs involves efficient capture and cross-presentation of tumor-associated antigens. However, tumors actively suppress capture and cross-presentation of tumor-associated antigen by DCs. Thus, we hypothesized that DCs deficient in LRP5/6 are more efficient in their capacity to capture and cross-present tumor-antigens to T cells. Given the enhanced antitumor immunity in LRP5/6 knockout mice, we determined whether this is due to efficient capture of tumor antigens. So, we implanted B16-GFP tumor cells in LRP5/6 knockout and control mice followed by analysis of DCs in the TDLN on day 7-post tumor implantation. Consistent with our previous data, we observed a significant increase in the frequencies of CD11c+ GFP+ DCs in the TDLN from LRP5/6 knockout mice compared to WT mice (Fig. 5A, B). Next, we determined whether activation of canonical Wnt-signaling in DCs limits cross-presentation of tumor antigens to CD8+ T cells. To test this, we transfected the CFSE-labeled naive OT-1 Thy1.1 CD8+ T cells into LRP5/6 knockout mice and WT-FM mice bearing B16-OVA tumors. We noted a significant increase in the proliferation of the progeny of CFSE labeled OT-1 Thy1.1 CD8+ T cells in LRP5/6 knockout mice compared to WT-FM mice (Fig. 5C, D). Likewise, deletion of LRP5/6 in DCs resulted in significant increase in the proliferation of CFSE labeled OT-1 Thy1.1 CD4+ cells (Fig. 5 E, F). Next, we determined whether the enhanced T cell proliferation observed in LRP5/6 knockout mice is due to increased migration of DCs to the TDLN. We noted a marked increase in the frequency of CD11c+ DCs in the TDLN of LRP5/6 knockout mice compared to WT mice (Fig. 5G). However, we observed similar levels of MHC class II expression in TDLN CD11c+ DCs isolated from WT and LRP5/6 knockout mice (Fig. 5H). This observation is consistent with previous studies suggesting that the LRP5/6 pathway is not critical for DC maturation. Collectively, these data suggest that activation of canonical Wnt-LRP5/6 signaling in TME DCs suppresses its migration, efficient capture of tumor-associated antigens and cross-priming of CD8+ T cells.

**Pharmacological inhibition of the LRP5/6 signaling axis enhances antitumor immunity against tumors**

To explore the above findings in a clinically relevant model, we examined the effects of pharmacological inhibition of binding of Wnt-ligands to cognate Fzd receptors using PORCN inhibitor III (IWP-L6). Porcupine (POCRN) is a membrane-bound O-acetyltransferase enzyme that palmitoylates Wnts, which is critical for its interactions with co-receptors LRP5/6 and Fzd receptors. Treatment of mice with established B16-OVA tumors or EL4-OVA tumors with IWP-L6 delayed tumor growth compared to untreated mice (Fig. 6A, B). In addition, IWP-L6 treatment resulted in significant increases in the frequencies of TNFα+CD8+, GzmB+ CD8+, IFNγ+ CD8+ and IFNγ+CD4+ effector T cells infiltrating the tumor (Fig. 6C–E). In contrast, blocking Wnt-signaling resulted in significant decrease in the frequencies of Foxp3+ Tregs and IL-10+ Tr1 cells within the tumors (Fig. 6E). Furthermore, the ratio of...
effectector T cells to Treg cells was markedly increased within the tumors of treated mice compared to the control mice (data not shown).

Next, we tested whether IWP-L6 treatment enhances DCs ability to capture and cross-present tumor antigens to CD8+ T cells. Like deletion of LRP5/6 in DCs, blocking Wnt-signaling resulted in significant increase in the frequency of CD11c+ GFP+ DCs in the TLDN of treated mice implanted with B16-GFP tumors (Fig. 6 F, G). Similarly, we observed robust proliferation of CFSE-labeled OT-I Thy1.1 CD8+ T cells in LRP5/6ΔDC mice compared to WT-FL mice (Fig. 6H, I). Furthermore, DCs isolated from TDLNs of treated mice expressed significantly higher levels of inflammatory cytokines IL-6, TNF-α, IL-12p19 and IL-12p35 and lower levels of IL-10, Aldh1a2 and TGF-β1 (data not shown). Consistent with this observation, IWP-L6 treatment of B16-OVA tumor-bearing mice that had received OT-I Thy1.1 CD8+ T cells showed significant increases in the frequencies of OT-II+ Foxp3+ Tregs and OT-II+ IL-10+ Tr1 cells within the tumors (data not shown). In line with these observations, TDLN lymphocytes from treated mice co-cultured with DCs pulsed with B16 tumor extract produced significantly higher levels of IFNγ and TNF-α and lower levels of IL-10 compared to TDLN lymphocytes from control mice (Fig. 6K). We confirmed these findings using another POCRN inhibitor, C59.29 Like IWP-L6 treatment, treatment of tumor-bearing mice with C59 delayed tumor growth (data not shown). Collectively, these data demonstrate that blocking the Wnt-LRP5/6 pathway promotes strong tumor-specific T cell responses by regulating DC function.

Discussion

Aberrant Wnt-signaling occurs in many tumors. However, little is known about the role of Wnts in antitumor immunity. The current study demonstrates for the first time that Wnts in the TME directly drive immune suppression by inducing regulatory DCs and limiting antitumor immunity. This is due to the role of canonical Wnt-signaling by regulating DCs ability to
capture tumor-associated antigens and its migration to TDLN. Furthermore, Wnts also limit efficient cross priming of CD8\(^+\) T cells by suppressing the expression of inflammatory cytokines and promoting the induction of regulatory factors such as IL-10, RA and TGF-\(\beta\). This resulted in enhanced regulatory T cell responses and decreased effector T cell responses to tumors. In addition, pharmacologically blocking the interaction of Wnts with LRP5/6 and FZD resulted in delayed tumor growth with enhanced antitumor immunity. Collectively, these findings support the hypothesis that Wnts in the TME induces regulatory DCs, which drives regulatory T cell responses and confines antitumor immunity. Hence, this pathway constitutes a new target for cancer immunotherapy.

The canonical Wnt-signaling that activates the \(\beta\)-catenin/TCF pathway is associated with many human tumors, but the focus of most research has been on the effects of this pathway on the tumor cells themselves.\(^{10-12}\) Wnt ligands in the TME could also initiate paracrine signaling within the immune cells and regulate host antitumor immunity.\(^{30}\) Our present study shows that deletion of LRP5/6 in DCs or blocking Wnt-signaling in mice results in delayed tumor growth with enhanced immune responses. Wnts activate both \(\beta\)-catenin-dependent and -independent signaling pathways in DCs.\(^{4}\) We, as well as others, have shown that tumors activate \(\beta\)-catenin in DCs and promote immune suppression by inducing regulatory T cell response while suppressing T effector cell responses.\(^{20-23}\) Ex \(\text{vivo}\) conditioning of DCs with Wnt ligands such as Wnt3a, Wnt5b and Wnt16 activates \(\beta\)-catenin pathway and program DCs to a regulatory state.\(^{4,15,23,26}\) Similarly, DCs isolated from melanoma patients displayed a tolerogenic phenotype with increase in the expression and activation of \(\beta\)-catenin.\(^{31}\) In addition to DC-intrinsic LRP5/6-signaling, active Wnt/\(\beta\)-catenin signaling within melanomas also promotes immune suppression.\(^{32,23}\) Thus, our data indicates that signaling initiated by Wnts in the TME activates the \(\beta\)-catenin/TCF pathway in DCs, which suppresses host antitumor immune responses and promotes tumor growth.

The normal initiation and activation of a robust antitumor immune response is associated with migration, efficient capture and presentation of tumor-associated antigens by DCs to T cells.\(^{8,34}\) It is well documented that immune regulatory factors in the TME suppress this process.\(^{6,35}\) The present study shows that canonical Wnt-signaling in DCs plays an important role in limiting DC migration and its ability to the uptake of tumor antigens. Deletion of LRP5/6 in DCs enhanced its ability to capture and present tumor antigens to T cells. Likewise, our experiments also show that blocking Wnts’ interaction with cognate receptors enhances the capture and cross-presentation of tumor antigens. In addition, deletion of LRP5/6 in DCs or blocking Wnt-signaling resulted in increased migration of DCs to TDLN. In line with this observation, a recent study has elegantly shown that active Wnt/\(\beta\)-catenin signaling in tumors suppresses the recruitment of DCs and T cells.\(^{32}\) However, it remains to be determined how DC-intrinsic LRP5/6-signaling regulates its migration to TDLN and tumors. Thus, our study identifies that canonical Wnt-signaling regulates DC migration and its ability to capture tumor-associated antigens.

Lastly, the cytokines produced by DCs dictate the type of T helper cell differentiation and adaptive immune responses against tumors.\(^{8,35}\) DCs in the TME are regulatory and they express immune regulatory molecules such as IDO, IL-10, TGF-\(\beta\) and RA that drive regulatory T cell responses and suppress T effector cell differentiation.\(^{5,6}\) Our data shows that either deletion of LRP5/6 or blocking LRP5/6 signaling in DCs programmed them to an inflammatory state, resulting in increased antitumor immunity. This was due to high levels of inflammatory cytokines such as IL-6, TNF-\(\alpha\), IL-1\(\beta\), IL-12p40 and IL-12p70 by DCs that are key in driving T effector cell differentiation and expansion. In contrast, DCs deficient in LRP5/6 expressed low levels IL-10, TGF-\(\beta\)1 and RA. In addition, parallel work has shown that Wnts in the TME induces IDO expression in DCs.\(^{23}\) In settings of inflammation, Wnt-signaling in DCs acts as a counter regulatory mechanism to suppress pathological chronic inflammation and restores homeostasis.\(^{13, 18, 25, 30, 35}\) However, we now show that in transplantable tumor settings, the same pathway in DCs drives pathological immune suppression and limits antitumor immunity. Thus, our findings show that canonical Wnt-signaling in DCs promotes immune suppression by regulating the expression of inflammatory and anti-inflammatory factors.

Our study also reveals a novel mechanism by which tumors promote immune suppression in the host. We have identified that the canonical Wnt pathway in DCs is a key regulator of immune tolerance to tumors. Disruption of this pathway altered DC function in the TME, resulting in enhanced host antitumor immune responses. These results represent an important advancement in our understanding of how Wnt-ligands in the TME can regulate host antitumor immunity and provide insight into potential immunotherapeutic strategies against tumors.

**Materials and methods**

**Mice**

C57BL/6 male mice of 6 to 12 weeks of age were purchased from The Jackson Laboratory (Bar Harbor, ME). OT-I\(\text{Thy1.1}(\text{Rag2}\text{-}\text{flox})\), OT-IIThy1.1 (Rag2\(\text{-}\text{flox}\)), LRP5 floxed,\(^{46}\) LRP5/6 floxed,\(^{36}\) LRP5/6 floxed mice,\(^{14}\) DC-specific deletion of LRP5, LRP6 and LRP5/6 (LRP5/6\(\Delta\text{DC})\),\(^{14}\) TCF/lymphoid enhancer factor (LEF)-reporter mice\(^{37}\) were bred on-site. All the mice were housed under specific pathogen-free conditions in the Laboratory Animal Services of Georgia Regents University. Animal care protocols were approved by the Institutional Animal Care and Use Committee of Georgia Regents University.

**Antibodies and reagents**

Antibodies against mouse CD4\(^+\) (GK1.5), CD8a (53–6.7), CD45 (30-F11), Foxp3 (FJK-16s), IL-10 (JES-16E3), CD11c (N418), CD90.1 (HS51), V\(\alpha\)2 TCR (B20.1), V\(\beta\)5.1/5.2 TCR (MR9-4), IL-17 (TC11-18H10), IFN\(\gamma\) (XMG1.2), TNF-\(\alpha\) (MP6-XT22), and Granzyme B (GB11) were purchased from eBioscience. Non-phospho active \(\beta\)-catenin antibody was obtained from Cell signaling technology. \(\beta\)-galactosidase (\(\beta\)-gal) antibody was purchased from Abcam. SKL2001, IWP-L6, C59 were obtained from Calbiochem and were dissolved in DMSO (10 mg/mL), and CFSE (Biolegend) was dissolved in
DMSO (10 mM). Wnt2b, 3a, 5a, 5b, and 16b were purchased from R&D systems. OVA (ISQVHAAHAEGAR) peptide was purchased from AnaSpec.

**Tumor model and in-vivo treatment**

Age-matched littermate controls were used for tumor studies. B16 melanoma cells expressing ovalbumin (B16-OVA), were provided from Dr David Munn (Georgia Regents University, Augusta, GA), LLC cells were obtained from Dr Andrew L. Mellor (Georgia Regents University, Augusta, GA), EL4 thymoma cells expressing ovalbumin (EL4-OVA) was purchased from the American Type Culture Collection, and B16-Green Fluorescent Protein (GFP) tumor cells were established by transducing parental B16 cells with recombinant lentivector containing GFP and selection with blasticidin by Dr Yukai He’s lab (Georgia Regents University, Augusta, GA). To establish tumor models, 5 × 10^5 MO4, EL4, LLC or B16-GFP cells were injected subcutaneously (s.c.) into mice in the shaved flank region. The tumor growth was monitored every 3 d by measuring the perpendicular diameters. The tumors were excised and the TDLNs were collected for various analyses. To establish the TDLNs were collected for various analyses. To study the tumor-bearing mice were administered with 4 g/mouse) or antibody against mouse CD4 (500 µg/mouse) on the same day of tumor inoculation and then on day 15 after tumor inoculation. In some experiments, tumor-bearing mice were administered with 4 × 10^6 CD8^+ T cells from OT-I TCR transgenic mice or CD4^+ T cells from OT-II TCR transgenic mice with or without CFSE (10 mM) labeling by intravenous (i.v.) route on day 9. Five days later, tumors and TDLNs were removed and stained with antibodies and analyzed by flow cytometry.

**Cell cultures**

CD11c^+ splenic DCs (2 × 10^5) enriched from TCF/LEF-reporter mice were isolated by FACS sorting and treated with Wnt2b, Wnt3a, Wnt5a, Wnt5b, Wnt16b (500 ng/mL) for 18 h, and then the cells were collected and analyzed by flow cytometry. In some experiments, purified CD11c^+ DCs (2 × 10^5) from LRP5/6^-/- mice and control-FL mice were treated with Wnt2b, Wnt3a, Wnt5a, Wnt5b, Wnt16b (500ng/mL) for 10 h and washed three times with medium, followed by co-culture with naive CD25^-CD4^+ OT-II T cells (1 × 10^5) in 200 µL RPMI complete medium containing OVA peptide (2 µg/mL) and TGFβ (1 ng/mL) in 96-well round-bottomed plates. After 5 d, cells were collected and analyzed by flow cytometry.

**Flow cytometry analysis**

To measure cytokines, single-cell suspensions from the TDLN or tumor were ex vivo stimulated with phorbol 12-myristate 13-acetate (PMA)/Ionomycin and Brefeldin A/monesin (eBioscience) for 6 h at 37°C, and intracellular staining of IFNγ, TNFα and IL10 was performed. To measure FoxP3, Granzyme B, active β-catenin, or β-gal expression, corresponding Abs were added after permeabilization and fixation of cells. Flow cytometric analysis was performed using a FACS LSRII system (BD Biosciences), and the data were analyzed using FlowJo software.

**Real-time PCR**

CD11c^+ DCs purified from pooled TDLNs or tumors and Total RNA was isolated using the Omega Total RNA Kit according to the manufacturer’s protocol. cDNA was generated using the RNA to cDNA Ecoky Premix Kit (Clontech) according to the manufacturer’s protocol. cDNA was used as a template for quantitative real-time PCR using SYBR Green Master Mix (Roche) and gene-specific primers. PCR analysis was performed using a MyiQ5 Icycler (BioRad). Gene expression was calculated relative to Gapdh.

**Statistical analyses**

Statistical analyses were performed using GraphPad Prism software. An unpaired one-tailed Student’s t test was used to determine statistical significance for tumor areas, mRNA expression levels, Treg percentages, Granzyme B expression and cytokines released by various cell types between different groups. A p value less than 0.05 (‘) was considered to be significant, a p value less than 0.01 (**) was considered to be very significant, and a p value less than 0.001 (***) was considered to be extremely significant.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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