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β-Catenin Promotes Regulatory T-cell Responses in Tumors by Inducing Vitamin A Metabolism in Dendritic Cells

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Abstract

Tumors actively suppress antitumor immunity, creating formidable barriers to successful cancer immunotherapy. The molecular mechanisms underlying tumor-induced immune tolerance are largely unknown. In the present study, we show that dendritic cells (DC) in the tumor microenvironment acquire the ability to metabolize vitamin A to produce retinoic acid (RA), which drives regulatory T-cell responses and immune tolerance. Tolerogenic responses were dependent on induction of vitamin A–metabolizing enzymes via the β-catenin/T-cell factor (TCF) pathway in DCs. Consistent with this observation, DC-specific deletion of β-catenin in mice markedly reduced regulatory T-cell responses and delayed melanoma growth. Pharmacologic inhibition of either vitamin A–metabolizing enzymes or the β-catenin/TCF4 pathway *in vivo* had similar effects on tumor growth and regulatory T-cell responses. Hence, β-catenin/TCF4 signaling induces local regulatory DC and regulatory T-cell phenotypes via the RA pathway, identifying this pathway as an important target for anticancer immunotherapy. *Cancer Res; 75(4); 656–65. ©2015 AACR.*

Introduction

Tumors promote immune tolerance, and dendritic cells (DC) play an important role in this. Although the molecular mechanisms of immune suppression by DCs are not fully understood, the ability of DCs to induce and activate regulatory T cells (Treg) is involved (1–5). Retinoic acid (RA), an active metabolite of vitamin A, regulates a broad array of immune responses (6, 7). Accumulated evidence suggests an important role for RA in immune tolerance in the gut by regulating the functions of antigen-presenting cells (APC), and by promoting the induction and activation of Treg (6, 7). A recent study has shown that tumor microenvironment (TME) contains high levels of RA, and APCs are major producers of RA (8). However, its role in Treg induction and activation in response to tumor-induced immune tolerance is not known. Moreover, molecular mechanisms whereby tumors induce APCs to produce RA remain poorly understood. So, we hypothesized that tumors, through DCs, exploit the RA pathway as a mechanism of immune evasion.

β-Catenin is a transcriptional cofactor that, upon activation, interacts with several family transcription factors, such as T-cell factors (TCF; ref. 9), PPARγ (10, 11), Foxo (12, 13), vitamin D receptor (VDR; refs. 12, 14), IRF3 (15), etc. Upon interaction with transcription factors, β-catenin can either promote or suppress the expression of target genes (9, 13, 14). Aberrant β-catenin signaling is associated with cancer development, progression, and even metastasis (9, 16, 17). A recent study has demonstrated that tumors activate β-catenin in APCs including DCs (18). However, its role in the induction and activation of Treg responses to tumor is not known. In addition, the downstream mediator of β-catenin signaling in DCs that drives immune tolerance to tumors remains poorly understood. In our previous study with intestinal DCs, we have shown that the β-catenin pathway programs DCs to a regulatory state and promotes immune tolerance to commensal microbiota (19). So, we hypothesize that tumors promote immune tolerance by activating the β-catenin/TCF pathway in DCs to induce RA, which drives regulatory T-cell responses.

In this study, using murine tumor models, we show that tumors program DCs to produce RA, which promotes immune suppression by inducing regulatory T-cell responses. This is mediated through the induction of vitamin A–metabolizing enzymes via the β-catenin/TCF pathway in DCs, which in turn drives regulatory T-cell responses and suppresses T-cell effector response.

Materials and Methods

Mice

C57BL/6 male mice of 6- to 12 weeks of age were purchased from The Jackson Laboratory. OT-II (Rag $2^{-/-}$) mice were purchased from Taconic. TCF/lymphoid enhancer factor (LEF)–reporter mice (20), β-catenin–floxed mice (21), and CD11c-cre

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(22) mice were originally obtained from The Jackson Laboratory and bred on-site. β-Catenin–floxed mice were crossed with transgenic mice expressing Cre recombinase under the control of a CD11c promoter, to generate mice lacking β-catenin in DCs (β-cat$^{\Delta DC}$). Successful cre-mediated deletion was confirmed by PCR and protein expression analyses as previously described (19). TCF4-floxed mice (23) were crossed to CD11c-cre mice to generate mice with DCs deficient in TCF4 (TCF4$^{\Delta DC}$), and successful cre-mediated deletion was confirmed by PCR (19). 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 (clone FJK-16s), IL10 (JES5-16E3), CD11c (clone N418), I-A^b (clone 25-9-17), CD90.1 (HIS51), V alpha 2 TCR (B20.1), V beta 5.1/5.2 TCR (MR9-4), IFNγ (XMG1.2), CD80 (16-10A1), CD86 (GL1), CD274 (MH5), and CD273 (TY25) were purchased from eBioscience. Nonphospho active β-catenin, β-catenin, and TCF4 antibodies were obtained from Cell Signaling Technology. β-Galactosidase (β-gal) antibody was purchased from Abcam. Retinol, all-trans retinoic acid (ATRA; Sigma-Aldrich), retinoid acid receptor antagonists LE135 (Tocoris), LE540 (Wako), Citral (Sigma-Aldrich), JW55 (Tocris), and XAV 939 (Tocris) were dissolved in DMSO (1 mmol/L). $OVA_{323–339}$ (ISQVHAAHAEINEAGR) peptide was purchased from Anaspec.

Plasmids, cell culture, transient transfection, and reporter assay

Raldh2 promoter luciferase construct was kindly provided by Dr. Ofelia M. Martínez-Estrada (24). Wild-type (WT) *β-catenin*, dominant-negative *β-catenin*, and active *β-catenin* plasmids were provided by Dr. Zuoming Sun (City of Hope). WT-*TCF4* and dominant-negative *TCF4* plasmids were from Addgene. 293T cells were cultured in DMEM supplemented with 10% FBS, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin. Cells ($2–3 \times 10^5$ in each well of a 24-well plate) were transfected with the *Raldh2* promoter reporter plasmid (100 ng) and expression vectors (500 ng) by the Lipofectamine method (Invitrogen). The total amount of transfected DNA was kept constant by adjusting the amount of the empty vector. Cells were collected after 24 hours and lysed in 200 μL reporter lysis buffer, and luciferase activities were measured by the Luciferase system, according to the manufacturer's instructions (Promega), and normalized against *Renilla* luciferase activities. "Fold Induction" represents normalized luciferase activity divided by the result of reporter-only groups.

Tumor model and *in vivo* treatment

Age-matched littermate control mice were used for tumor studies. B16 melanoma expressing ovalbumin, clone MO4, was obtained from Dr. David Munn (Georgia Regents University, Augusta, GA; ref. 25), and EL4 lymphoma expressing ovalbumin, clone E.G7-OVA (EG7), was obtained from the American Type Culture Collection. To establish tumors, 5×10^5 MO4 and EG7 cells were injected s.c. into mice in the shaved flank region. The tumor growth was monitored every 3 days by measuring the perpendicular diameters. The experiments were terminated when tumor size reached area greater than 2 cm^2. The tumors were excised, and tumor-draining lymph nodes (TDLN) were collected for various analyses. To study the effect of disulfiram, ATRA, JW55, and XAV 939 *in vivo*, tumor transplanted C57BL/6 or β-catenin$^{\Delta DC}$ or respective littermate control mice were injected i.p. with disulfiram (5 mg/kg) or ATRA (2.5 mg/kg), JW55 (2 mg/kg), or XAV 939 (2 mg/kg) every 3 days on days 5, 8, 11, and 14 after tumor implantation. Alternatively, mice were treated from day 10 followed by days 13, 16, and 19 after tumor transplantation with XAV939 or with citral (2 mg/mL) in water from day 5 after tumor implantation until day 17 after tumor inoculation. In some experiments, tumor-bearing mice were administered with these treatments, and on day 9, 4×10^6 sorted CD4$^+$CD25$^-$ T cells from Rag$^{-/-}$ OT-II TCR transgenic mice were injected by i.v. route. Five days later, tumors and TDLNs were removed and stained with antibodies and analyzed by flow cytometry.

Cell cultures

CD45$^+$CD11c$^+$ cells from TDLN or tumor were isolated by FACS sorting and cocultured with naïve OT-II (CD4$^+$CD25$^-$) T cells in the presence of OVA peptide (2 μg/mL), TGFβ (1 ng/mL), and IL2 (5 ng/mL). After 5 days, cells were stained and analyzed for CD4 and Foxp3 expression. In some experiments, purified CD11c$^+$ DCs (2×10^4) cells were treated with disulfiram (Sigma-Aldrich; 100 nmol/L), retinol (500 nmol/L), ATRA (50 nmol/L), or LE135/LE540 (1 μmol/L) as described in our previous study (26) and followed by coculture with naïve 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 days, cells were collected and analyzed by flow cytometry.

Flow cytometry analysis

To measure cytokines, single-cell suspensions from spleen, TDLN, or tumor were *ex vivo* stimulated with phorbol 12-myristate 13-acetate (PMA)/Ionomycin and BrefedinA/monesin (eBioscience) for 6 hours at 37°C, and intracellular staining of IFNγ and IL10 was performed. To measure Treg or β-catenin or β-gal expression, corresponding Abs were added after permeabilization and fixation of cells (27). To detect aldehyde dehydrogenase (ALDH) activity, we used the ALDEFLUOR Kit (Stemcell) following the manufacturer's recommendations (27). Flow cytometric analysis was performed using a FACS LSRII system (BD Biosciences), and the data were analyzed using FlowJo software.

Real-time PCR

Total mRNA was isolated from purified splenic, lymph node, and tumor CD11c$^+$ DCs using the Omega Total RNA Kit according to the manufacturer's protocol. cDNA was generated using the RNA to cDNA Ecodry 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 (19, 26). 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 *t* test was used to

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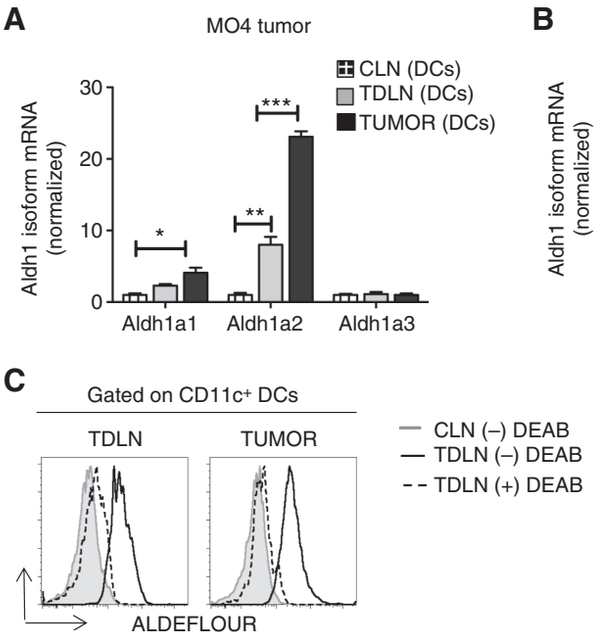

Figure 1.
DCs express vitamin A–metabolizing enzymes in response to tumor. Real-time PCR analysis of mRNA levels of *Aldh1* isoforms (*Aldh1a1*, *Aldh1a2*, *Aldh1a3*) expression in CD11c$^+$ DCs enriched from TDLNs, CLNs, and tumors pooled from WT mice on day 9 after tumor inoculation MO4 (A) and EG7 (*n* values are represented in triplicates from two independent experiments, with pooled DCs obtained from 5 mice per experiment; B). C, *ex vivo* analysis of ALDH activity in CD11c$^+$ DCs cells enriched from TDLNs, CLNs, and tumors from WT mice ($n =$ cells were pooled from 5–6 tumor-bearing mice). DEAB, a specific inhibitor of ALDH, was used as control for background fluorescence. Data are representative of at least two independent experiments. Error bars, mean values $\pm$ SD. Statistical levels of significance were analyzed by the Student *t* test (unpaired). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

determine statistical significance for tumor areas, mRNA expression levels, Treg percentages, 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.

Results

Tumors induce expression of RA-synthesizing enzymes in DCs

TME and TDLNs have elevated levels of RA, and tumor APCs act as a major producer/source of RA (8). Within cells, RA is produced from vitamin A (retinol) via a two-step enzymatic pathway where retinol is first oxidized to retinaldehyde (retinal) followed by conversion to RA (7). First, we investigated whether DCs from TME and TDLNs express enzymes that are critical for RA biosynthesis. RT-PCR analysis showed that control lymph node (CLN) DCs and TDLN DCs expressed similar levels of *alcohol dehydrogenase (Adh)1* and *Adh5*, suggesting *Adh* expression in DCs is not regulated in response to tumor (data not shown). Interestingly, however, TDLN DCs and tumor DCs showed a significant increase in the expression of *Aldh1a1* and *Aldh1a2* isoforms compared with the CLN DCs (Fig. 1A and B). This was unexpected because unlike intestinal DCs, peripheral DCs do not express these genes under homeostatic conditions (7). To further validate these findings, we performed Aldh activity assay. TDLN DCs and TME DCs showed markedly increased Aldh activity compared with CLN DCs or DEAB (Aldh1-specific inhibitor)-treated DCs (Fig. 1C). With further analysis of DC subsets, we observed high levels of Aldh1 activity in TDLN CD11c$^+$CD11b$^-$ DCs and plasmacytoid DCs (pDC; data not shown). Taken together, these data indicate that DCs express high levels of Aldh1, a key enzyme in RA synthesis, in response to tumors.

TDLN DCs induce regulatory T-cell differentiation via an RA-dependent pathway

TME conditions DCs to acquire regulatory properties that mostly induce regulatory T-cell responses (1, 3, 4, 28). However, it is not known whether Treg activation and induction by DCs in the TME is dependent on RA. We hypothesized that Treg activation and induction by DC is dependent on RA. To test this, we evaluated the ability of TDLN DCs to promote differentiation of naïve CD4$^+$ T cells into Foxp3$^+$ Tregs in the presence of RA inhibitors. As shown in Fig. 2A, blocking Aldh1 activity with disulfiram (Aldh1 inhibitor; ref. 26) significantly reduced the ability of TDLN DCs to induce the differentiation of Tregs. Because TDLN DCs express Aldh1, we next determined whether these DCs could metabolize vitamin A (retinol) to RA, and promote Treg differentiation. Accordingly, addition of exogenous vitamin A (retinol) enhanced the conversion of CD4 T cells to Foxp3$^+$ Tregs, indicating that TDLN DCs can actively metabolize vitamin A to RA and drive Treg differentiation (Fig. 2A and B). Furthermore, addition of pan-RAR (retinoic acid receptor) inhibitors (LE540/LE135) to the cocultures markedly reduced the proportion of Tregs induced by TDLN DCs (Fig. 2A and B), suggesting that Treg induction is dependent on RA and the RAR–RXR pathway. Taken together, these data demonstrate that TDLN DCs can metabolize vitamin A to RA and can drive Treg differentiation.

To investigate the *in vivo* role of RA in Treg induction in response to tumor, we examined the effects of pharmacologic inhibition of Aldh1 activity with disulfiram as performed in our previous study (26). WT mice implanted with MO4 tumor cells were treated with or without disulfiram and followed by adoptive transfer of naïve OT-II CD4$^+$ T cells (Fig. 2C). Five days after adoptive transfer, the frequency of OVA-specific Foxp3$^+$CD4$^+$ Tregs in the tumor and TDLN was analyzed. First, we assessed whether treatment with disulfiram inhibited Aldh1 activity in TDLN DCs. Treatment of tumor-bearing mice

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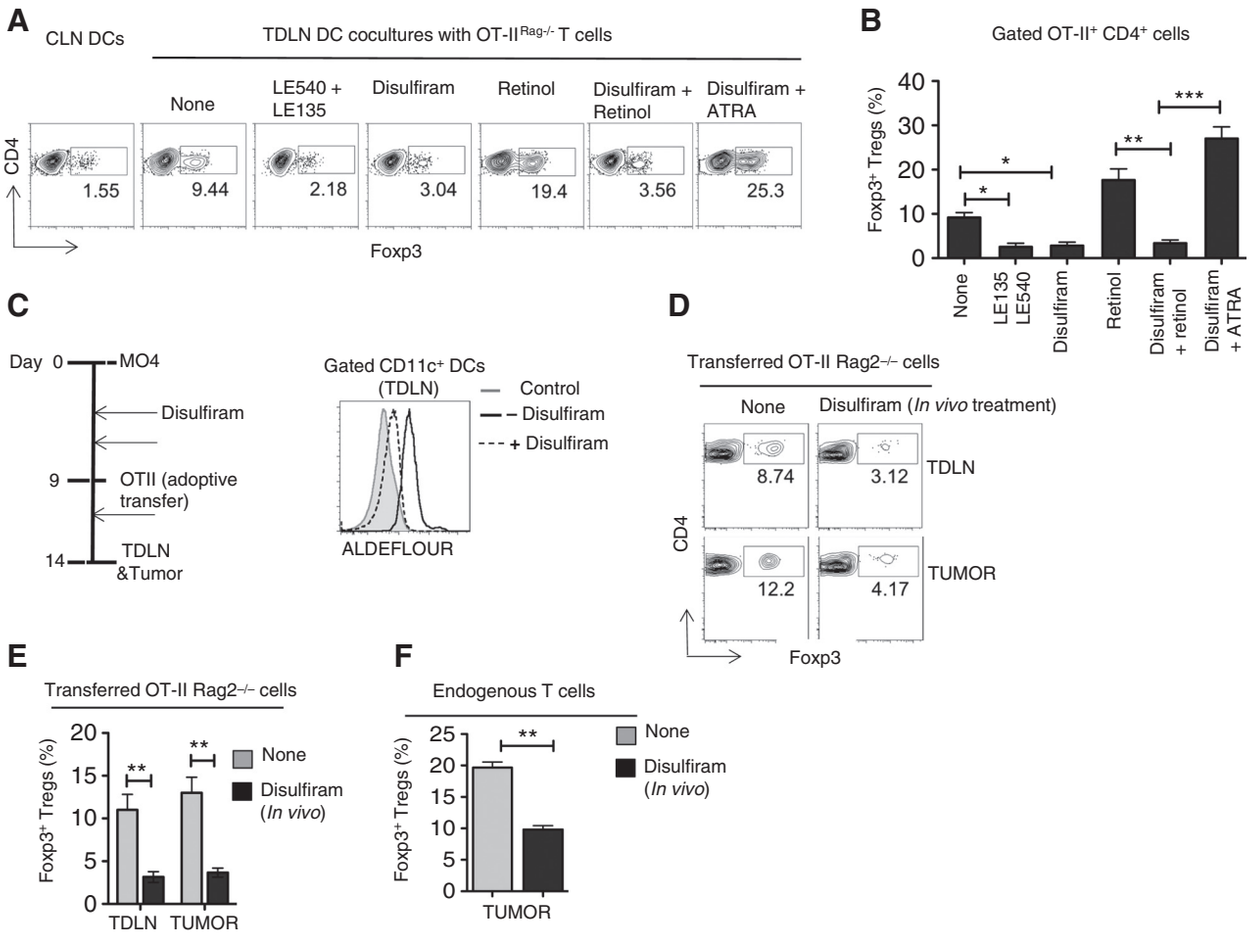

Figure 2.
RA in TME induces Foxp3$^+$ Tregs. A and B, CD11c$^+$ DCs were sorted from TDLNs and CLNs of MO4 tumor–bearing WT mice on days 9 to 11 after tumor inoculation and were treated with or without disulfiram (1 μmol/L). After 3 hours, DCs were washed and cocultured with CD4$^+$CD25$^-$ cells from Rag$^{-/-}$ OTII mice in RPMI1640 medium containing OVA peptide (2 μg/mL) and TGFβ (1 ng/mL), in the presence or absence of LE540+LE135 (1 μmol/L) or retinol (1 μmol/L) or ATRA (5 nmol/L) combination for 5 days. Representative FACS plots (A) and frequencies (B) of Foxp3$^+$CD4$^+$ T ($n = 3$; DCs were pooled from 5 to 6 mice, and experiment was repeated at least two times). C, *ex vivo* analysis of ALDH activity in CD11c$^+$ DCs enriched from TDLNs, CLNs, and tumors from WT mice treated with or without disulfiram on day 14 after tumor inoculation. DEAB, a specific inhibitor of ALDH, was used as control for background fluorescence. Data shown are representative histograms gated on CD45$^+$ CD11c$^+$ cells from one experiment of two independent experiments. DCs were pooled from 5 to 6 tumor-bearing mice for each experiment. D and E, MO4-bearing mice treated with or without disulfiram were adoptively transferred with OT-II T cells from Rag$^{-/-}$OTII mice on day 9 after tumor inoculation. After 5 days, FACS analysis was performed on CD45$^+$Vα2$^+$Vβ5.1/5.2$^+$ CD4$^+$ T cells from TDLNs and tumor tissues. D, data shown are percentage and representative dot plots of OVA-specific Foxp3$^+$CD4$^+$ T cells ($n = 3$ to 4 mice per experiment. Experiment was repeated two times). E, data are from experiment D, showing cumulative frequencies of adoptively OVA-specific Foxp3$^+$ CD4$^+$ T cells and showing mean values ± SEM ($n = 6$ to 8 mice). F, percentage of endogenous Foxp3+ CD4+ Treg cells in tumors isolated from WT mice treated with or without disulfiram ($n = 5$ mice). Error bars, mean values ± SEM. Statistical levels of significance were analyzed by the Student *t* test (unpaired) *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

with disulfiram significantly decreased the Aldh1 activity in TDLN DCs as compared with untreated control group (Fig. 2C). In addition, disulfiram treatment of tumor-bearing mice significantly delayed tumor growth in mice compared with control mice (Supplementary Fig. S1A). Next, we analyzed the differences in frequency of Foxp3$^+$ OT-II T cells treated with or without disulfiram. Blocking Aldh1 activity markedly reduced the frequency of induced Tregs both in TDLN and in tumor tissue compared with untreated mice (Fig. 2D and E). Moreover, disulfiram treatment also affected the frequency of endogenous Tregs within the tumor (Fig. 2F). These findings were further confirmed with citral, another Aldh1 inhibitor (Supplementary Fig. S1B and S1C; ref. 29). These observations demonstrate that pharmacologic blocking of vitamin A–metabolizing enzyme suppresses Treg differentiation and enhances antitumor immunity. Thus, RA is necessary for *in vivo* induction of Tregs in response to tumors.

β-Catenin/TCF4 is critical for the expression of vitamin A–metabolizing enzymes in DCs

Recent studies have shown that tumors activate β-catenin in immune cells including DCs (18, 30). Our previous study on intestinal DCs has shown that Aldh1a1 and Aldh1a2 are

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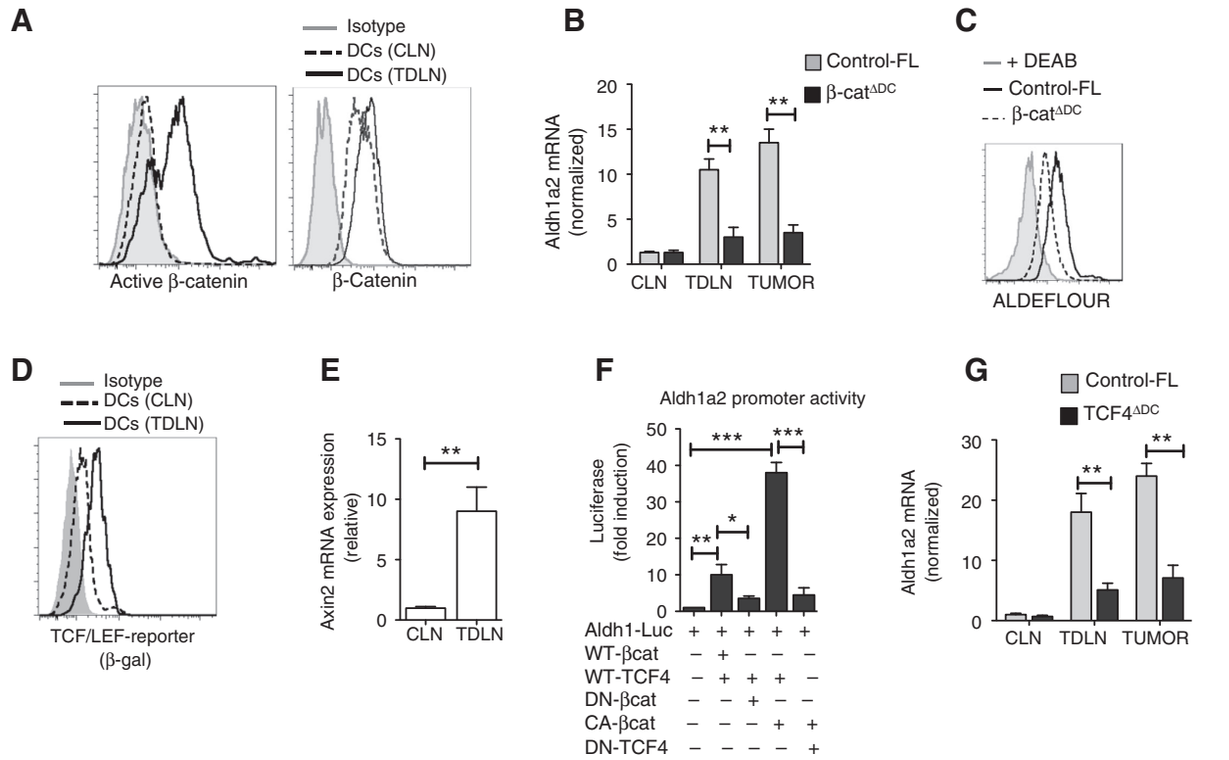

Figure 3.
β-Catenin/TCF4 pathway induces vitamin A–metabolizing enzymes in DCs. A, representative histograms showing active β-catenin (left) and β-catenin (right) expression in CD11c$^+$ cells from MO4 tumor TDLN and CLN of tumor-free C57BL/6 (WT) mice on day 9 after tumor inoculation. B and C, real-time PCR analysis of *Aldh1a2* expression from TDLNs, CLNs, and tumors ($n = 3$; B) and *ex vivo* analysis of ALDH activity in CD11c$^+$ DCs enriched from TDLNs from β-cat$^{\Delta DC}$ and control-FL mice (C). D, representative histograms showing β-gal expression in CD11c$^+$ cells from TDLNs and CLNs of *TCF/LEF-LacZ* reporter mice. E, *Axin2* mRNA expression analyzed by qRT-PCR in TDLNs and CLNs on day 9 after inoculation ($n = 4$). The result represents fold increase over the CLNs. F, *Aldh1a2* promoter activity in 293T cell after 24-hour transfection reporter plasmid alone or cotransfection with WT-*β-catenin* (WT-*βcat*), dominant-negative *β-catenin* (DN-*βcat*), active *β-catenin* (CA-*βcat*), WT-*TCF4*, or dominant-negative *TCF4* (DN-*TCF4*) expression vectors. The results are represented as fold activation relative to *Aldh1* promoter vector transfection control alone. G, real-time PCR analysis of *Aldh1a2* expression in CD11c$^+$ DCs enriched from TDLNs, CLNs, and tumors from TCF4$^{\Delta DC}$ and control-FL mice. Iso, isotype control. DCs were enriched and pooled from 5 to 6 tumor-bearing mice (A, C, and D). Each sample was done in triplicate; the average and SD are shown. The experiment was repeated at least two (B, E, and G) or three (F) times with similar results. Error bars, mean values ± SEM. Statistical levels of significance were analyzed by the Student *t* test (unpaired) *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

β-catenin target genes (19). We hypothesized that tumor-induced activation of the β-catenin pathway in DCs is critical for upregulation of RA-synthesizing enzymes. So, we assessed whether tumors activate β-catenin in DCs using an antibody that recognizes the active form of β-catenin. Consistent with a previous study (18), TDLN DCs showed increased levels of active β-catenin compared with CLN DCs or isotype control (Fig. 3A, right plot). However, both CLN DCs and TDLN DCs expressed a similar level of total β-catenin (Fig. 3A, left plot), suggesting that tumors induce the activation of β-catenin in DCs but do not affect its expression. Next, we examined whether induction of Aldh1 in TDLN DCs is dependent on the activation of β-catenin. In contrast with TDLN DCs from control-FL mice, DCs deficient in β-catenin (19) expressed significantly lower levels of Aldh1 in response to MO4 and EG7 tumors (Fig. 3B; Supplementary Fig. S2). Consistent with this observation, β-catenin–deficient TDLN DCs showed markedly reduced Aldh1 activity compared with respective control-FL TDLN DCs (Fig. 3C). Collectively, these data demonstrate that TME programs DCs to express Aldh1a2 in a β-catenin–dependent manner.

We next investigated the downstream signaling pathways through which β-catenin can induce Aldh1 expression in DCs. The TCF family of transcription factors is one of the main downstream mediators of β-catenin signaling (9), and DCs highly express the TCF4 isoform (31). So, we tested whether tumor-induced activation of β-catenin in DCs promotes *TCF/LEF*-dependent gene transcription using TCF/LEF β-gal reporter mice (19). TDLN DCs from the reporter mice showed strong *β-gal* expression compared with CLN DCs (Fig. 3D). Consistent with this, we also observed increased expression of the *β-catenin/TCF4* target gene, *Axin2*, in TDLN DCs compared with CLN DCs (Fig. 3E). Taken together, our data demonstrate that activation of β-catenin induces the expression of *TCF4* target genes in DCs in response to tumor.

Based on the above observation, we reasoned that TCF4 is the downstream mediator of β-catenin signaling in DCs that drives the expression of RA-synthesizing enzymes in response to tumor. Promoter analysis using consensus TCF-binding sites showed several potential TCF sites in the *Aldh1a2* promoter region with some near the transcription initiation regions. To further investigate transcriptional

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regulation of *Aldh1a2* by β-catenin/TCF4, 293T cells were stably transfected with a 830-bp *Aldh1a2*-promoter luciferase reporter plasmid (24) either alone or in combination with WT or mutant *β-catenin* or *TCF4* expression plasmids. Cotransfection with WT *β-catenin* and WT-*TCF4* plasmids resulted in 10- to 12-fold increase of *Aldh1a2* promoter activity (Fig. 3F). Likewise, expression of active *β-catenin* further increased promoter activity. In contrast, expression of a dominant-negative *β-catenin* or *TCF4* markedly decreased its activity (Fig. 3F). Consistent with these observations, deletion of TCF4 specifically in DCs resulted in decreased expression of *Aldh1a2* gene in response to tumor (Fig. 3G). Taken together, these results clearly demonstrate that TME induces expression of activated *β-catenin* in DCs, which interacts with TCF4 and promotes transcriptional activation of *Aldh1* gene expression.

DC-specific deletion of β-catenin limits regulatory T-cell induction and tumor growth

The preceding experiments showed that tumor-associated DCs produce RA and drive regulatory T-cell differentiation. So, we hypothesized that tumor-induced activation of the β-catenin/TCF pathway in DCs induces regulatory T-cell responses and promotes immune tolerance. To test this, we monitored MO4 and EG7 tumor growth over time in mice that specifically lack β-catenin in DCs (19). Deletion of β-catenin in DCs (β-cat$^{\Delta DC}$) significantly reduced tumor growth compared with that seen with WT mice (Fig. 4A and B). Next, we examined the effector phenotype of tumor-infiltrating lymphocytes isolated from both groups of mice. Interestingly, we observed a significant decrease in the frequency of Foxp3$^+$CD4$^+$ Tregs, IL10$^+$CD4$^+$ Tr1 cells, and IL10$^+$CD8$^+$ T cells in tumors of β-cat$^{\Delta DC}$ mice compared with controls (Fig. 4C and D). In contrast, melanomas isolated from β-cat$^{\Delta DC}$ mice contained a higher frequency of IFNγ$^+$CD4$^+$ and IFNγ$^+$ CD8$^+$ T cells compared with melanomas of control mice (Fig. 4E and F). These findings were further confirmed with EG7 tumor (Supplementary Fig. S3A). Collectively, these results show that tumor-mediated activation of β-catenin in DCs promotes regulatory T-cell responses and limits antitumor immunity.

Next, we wished to test whether enhanced antitumor immunity and reduced regulatory T-cell responses observed in β-cat$^{\Delta DC}$ mice are due to changes in DC maturation or activation. Phenotypic characterization of TDLN CD11c$^+$ DCs from β-cat$^{\Delta DC}$ mice expressed greatly enhanced levels of the activation markers CD80 and CD86, and reduced levels of coinhibitory molecules such as PDL1 and PDL2. These observations suggest that the β-catenin pathway also regulates the activation status of DCs. However, we observed similar levels of MHC class II expression in TDLN CD11c$^+$ DCs isolated from WT and β-cat$^{\Delta DC}$ mice (Supplementary Fig. S3B). This observation is consistent with previous studies suggesting that the β-catenin pathway is not critical for DC maturation (27, 32, 33). Taken together, these results show that tumor-mediated activation of β-catenin in DCs limits antitumor immunity at least in part

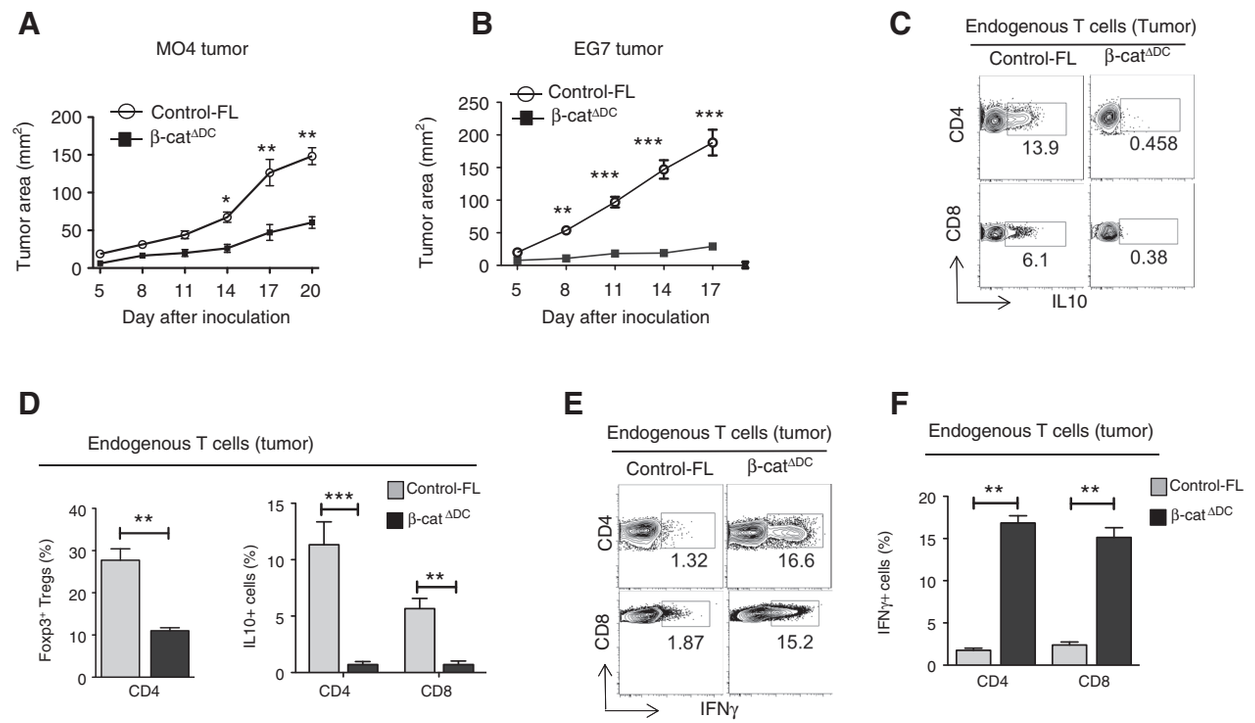

Figure 4.
DC-specific deletion of β-catenin enhances antitumor immunity in mice. A, MO4 melanoma progression in β-cat$^{\Delta DC}$ and control-FL mice ($n = 10$–12). B, EG-7 tumor progression in β-cat$^{\Delta DC}$ and control-FL mice ($n = 6$–8). C–F, representative dot plots and percentages of IL10$^+$CD4$^+$, Foxp3$^+$CD4$^+$, IL10$^+$CD8$^+$, IFNγ$^+$CD4$^+$, and IFNγ$^+$CD8$^+$ T cells isolated from MO4 melanoma in control-FL and β-cat$^{\Delta DC}$ mice on day 14 after inoculation ($n = 5$ to 6 mice). Data are representative of two independent experiments and show mean values ± SEM. Statistical levels of significance were analyzed by the Student *t* test (unpaired). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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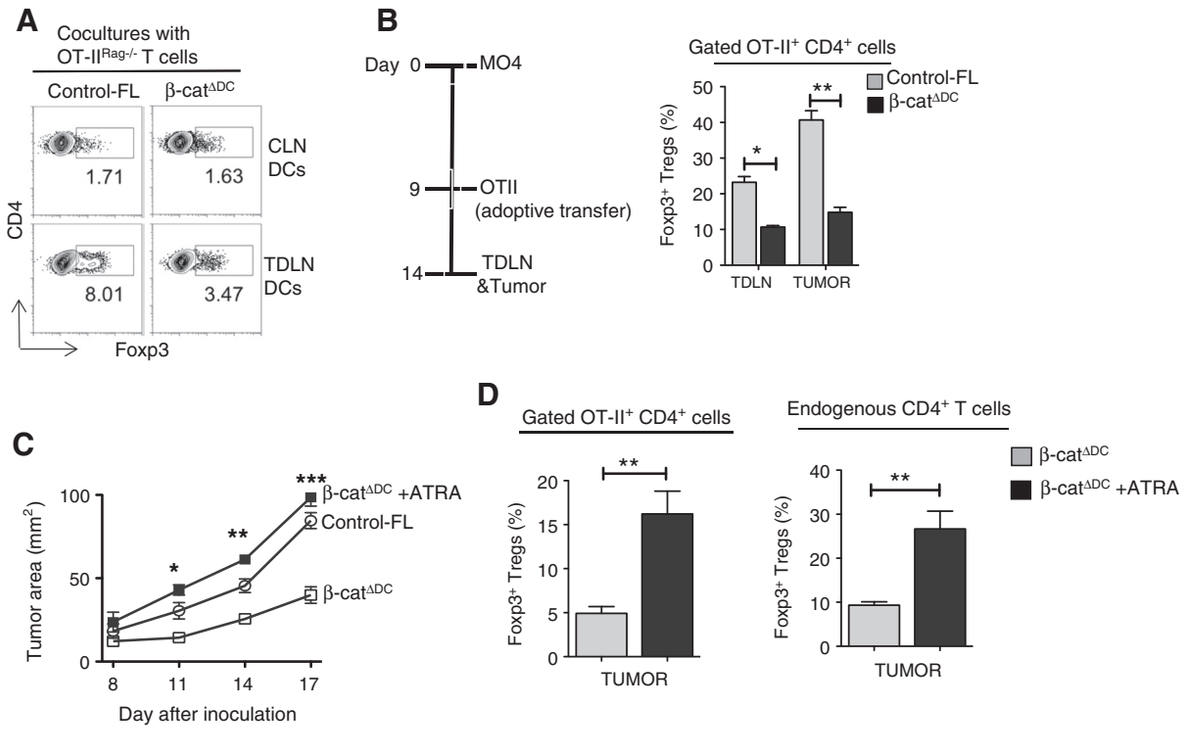

Figure 5.

Loss of β-catenin impairs DCs ability to induce Tregs differentiation, and ATRA treatment can restore it. A, $CD11c^+$ cells were sorted from CLNs (top) and TDLNs (bottom) of MO4 tumor–bearing β-$cat^{\Delta DC}$ and control-FL mice and were cocultured with $CD4^+CD25^-$ naïve T cells as described in Fig. 2A. Data are shown as percentage and representative dot plots of $Foxp3^+CD4^+$ T cells of one experiment of two independent experiments (DCs were pooled and enriched from 5 to 6 mice for each experiment). Each sample was done in triplicate. B, *in vivo* OT-II Treg differentiation in β-$cat^{\Delta DC}$ and control-FL mice was performed as described in Fig. 2D. Data, cumulative percentage of OVA-specific $Foxp3^+CD4^+$ T cells in TDLNs and tumors isolated from β-$cat^{\Delta DC}$ and control-FL mice ($n = 5$–6). C, the MO4 melanoma progression in β-$cat^{\Delta DC}$ mice treated with or without ATRA (2.5 mg/kg) every 3 days from day 1 after tumor inoculation. Data, mean tumor size and are cumulative, representative of two independent experiments β-$cat^{\Delta DC}$ versus β-$cat^{\Delta DC}$ +ATRA ($n = 6$–8 mice; combined results of two independent experiments are shown). D, *in vivo* OT-II Treg differentiation in β-$cat^{\Delta DC}$ treated with or without ATRA was performed as described in Fig. 2D. Percentage of OVA-specific $Foxp3^+CD4^+$ Tregs and endogenous $Foxp3^+CD4^+$ Tregs in TDLNs and tumors isolated from β-$cat^{\Delta DC}$ and control-FL mice treated with or without ATRA ($n = 6$ to 8 mice). Data, two independent experiments and show mean values ± SEM. Statistical levels of significance were analyzed by the Student *t* test (unpaired). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

by regulating the expression levels of coinhibitory and costimulatory molecules.

Tumor promotes regulatory DCs via a β-catenin/RA axis

The preceding results demonstrated that activation of β-catenin in DCs promotes regulatory T-cell response and limits T-cell effector response. So, we reasoned that tumor-induced activation of the β-catenin pathway imparts regulatory phenotype on DCs and promotes regulatory T-cell response. Therefore, we tested the ability of TDLN DCs isolated from control-FL and β-$cat^{\Delta DC}$ mice to promote differentiation of naïve $CD4^+$ T cells into $Foxp3^+$ Tregs. TDLN DCs from control-FL mice induced a higher frequency of $Foxp3^+$ Tregs compared with CLN DCs (Fig. 5A). In contrast, TDLN DCs from β-$cat^{\Delta DC}$ mice induced a lower frequency of $Foxp3^+$ Tregs compared with control TDLN DCs (Fig. 5A).

To extend these observations *in vivo*, we implanted MO4 melanoma cells in control-FL and β-$cat^{\Delta DC}$ mice, and on day 9, naïve OT-II $CD4^+$ T cells were adoptively transferred as shown in Fig. 5B. Consistent with *in vitro* results, the frequency of induced OVA-specific $Foxp3^+CD4^+$ Tregs was markedly reduced both in TDLN and in tumors of β-$cat^{\Delta DC}$ mice compared with control mice in response to tumor burden (Fig. 5B). Altogether, these data demonstrate that tumor-induced activation of β-catenin in DCs imparts regulatory phenotype and drives Treg differentiation.

The above results show that deletion of β-catenin in DCs results in loss of regulatory phenotype and limits DCs ability to drive Treg induction. Moreover, these DCs express low levels of RA-synthesizing enzymes. Therefore, we hypothesized that exogenous delivery of RA would promote regulatory T-cell differentiation and restore the tumor growth in β-$cat^{\Delta DC}$ mice. To test this, we challenged these mice with MO4 cells and then treated them with RA. As expected, tumor growth in the β-$cat^{\Delta DC}$ mice was delayed and lagged behind tumor growth seen with control mice (Fig. 5C). In contrast, RA treatment of β-$cat^{\Delta DC}$ mice accelerated tumor growth compared with the untreated mice (Fig. 5C). We then asked whether this accelerated tumor growth is associated with an increase in endogenous as well as antigen-specific Tregs. Injection of OT-II $CD4^+$ T cells into RA-treated β-$cat^{\Delta DC}$ tumor-bearing mice resulted in increased frequency of OVA-specific $Foxp3^+$ $CD4^+$ Tregs within tumors (Fig. 5D). We also observed marked increase in the frequency of endogenous $Foxp3^+$ $CD4^+$ Tregs within tumors (Fig. 5D). These data suggest that RA can induce Tregs and promote

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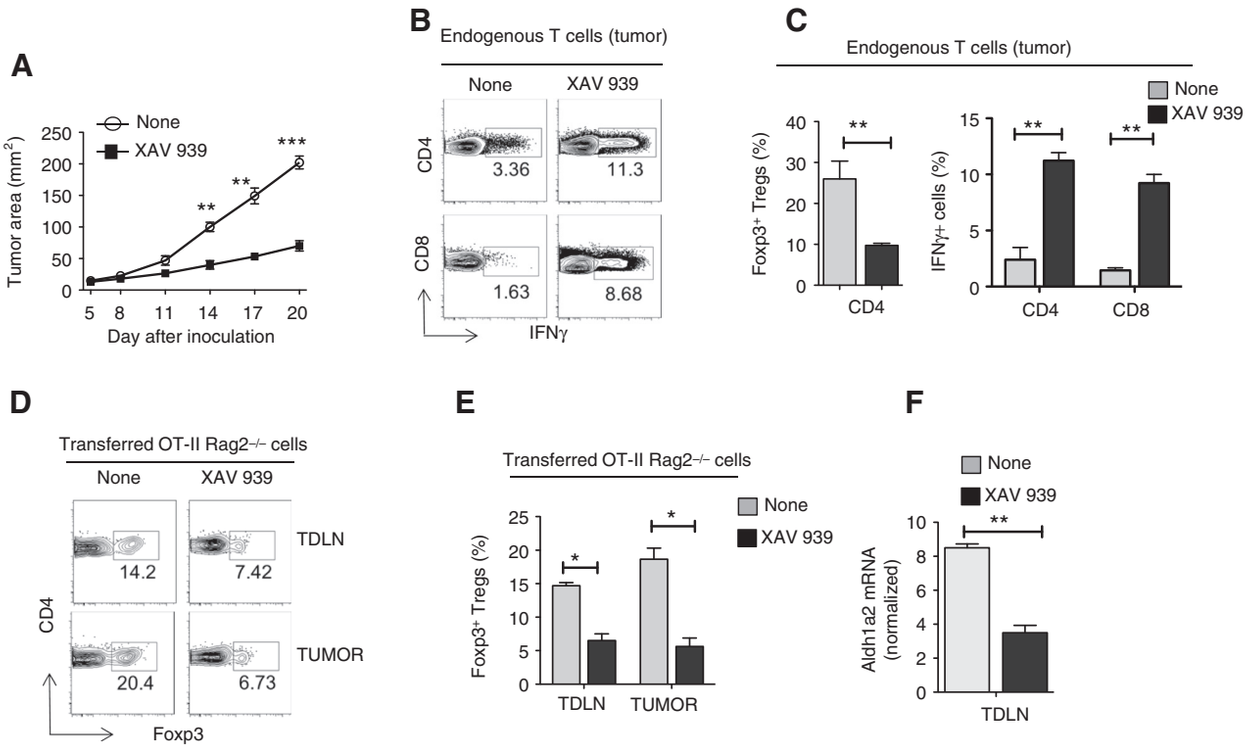

Figure 6.
Therapeutic effect of blocking the β-catenin pathway against established tumors. The MO4 melanoma progression in WT mice treated with or without XAV 939 (2 mg/kg) every 3 days from day 5 after tumor inoculation. A, data are mean tumor size and are cumulative, representative of two independent experiments ($n = 10$–12 mice; data show combined results of two independent experiments with 5 to 6 mice per experiment). B and C, representative dot plots and percentage of $IFN\gamma^+CD4^+$, $IFN\gamma^+CD8^+$, and $Foxp3^+CD4^+$ T cells isolated from MO4 on day 20 after inoculation. D and E, *in vivo* OT-II Treg differentiation in WT mice treated with or without XAV 939 was performed as described in Fig. 2D. Data are representative dot plots (D) and cumulative percentage (E) of induced OVA-specific $Foxp3^+CD4^+$ Tregs isolated from TDLN and tumor ($n = 5$ to 6 per sample). Data are representative of two independent experiments and show mean values ± SEM. Combined results of two separate experiments are shown, with 5 to 6 mice per group in each experiment. F, real-time PCR analysis of *Aldh1a2* expression in $CD11c^+$ DCs enriched from TDLNs from WT mice treated with or without XAV 939. Each sample was done in triplicate; the average and SD are shown. The experiment was repeated two times with similar results. Statistical levels of significance were analyzed by the Student *t* test (unpaired). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

tumor growth, further emphasizing the contribution of the β-catenin/TCF4-RA axis in immune tolerance to tumors.

Pharmacologic inhibition of the β-catenin/TCF pathway impairs regulatory T-cell induction and enhances antitumor immunity

To explore the above findings in a clinically relevant model, we examined the effects of pharmacologic inhibition of the β-catenin/TCF pathway with the tankyrase inhibitor, XAV 939 (34). Accordingly, treatment of melanoma or EG7 tumor–bearing WT mice with XAV 939 delayed tumor growth compared with untreated mice (Fig. 6A; Supplementary Fig. S4A). In addition, therapeutic treatment of mice with XAV 939 at day 10 after tumor transplantation, when the tumor volume is greater, also delayed the tumor growth (Supplementary Fig. S4B). Consistent with the reduced tumor growth, XAV 939 treatment resulted in significant decrease in numbers of Tregs and an increase in the frequencies of $IFN\gamma^+CD4^+$ and $IFN\gamma^+CD8^+$ effector T cells infiltrating the tumor (Fig. 6B and C; Supplementary Fig. S4C). Furthermore, the ratio of effector T cells to Tregs was markedly increased within the tumor of treated mice compared with the control mice (data not shown). Consistent with this observation, XAV 939 treatment of tumor-bearing mice that had received OT-II $CD4^+$ T cells by adoptive transfer resulted in the decrease in frequency and number of OVA-specific Tregs within tumors (Fig. 6D and E). Consistent with the reduced Treg induction, XAV 939 treatment markedly reduced the expression of vitamin A–metabolizing enzymes (Fig. 6F; Supplementary Fig. S4D). We confirmed these findings in using another β-catenin/TCF inhibitor, JW55 (35, 36; Supplementary Fig. S5). Collectively, these data demonstrate that blocking the β-catenin/TCF4 pathway suppresses Treg differentiation and enhances antitumor immunity.

Discussion

Tumors actively suppress immune responses, and this represents a fundamental barrier to cancer immunotherapy. DCs are important in this process, but the molecular mechanisms of immune suppression by DCs are not fully understood. The current study demonstrates for the first time that RA in the TME directly drives immune suppression by inducing regulatory T-cell responses. A key mechanism of this is through induction of vitamin A–metabolizing enzymes in DC via the β-catenin/TCF

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pathway, which in turn drives regulatory T-cell responses and suppresses T effector cell response. Pharmacologic intervention of vitamin A–metabolizing enzyme activity or the β-catenin/TCF pathway limits regulatory T-cell responses and enhances antitumor immunity. Collectively, these findings support the hypothesis that tumors induce DCs to produce RA by activating the β-catenin/TCF4 pathway, thereby promoting immune tolerance. Hence, this pathway constitutes a new target for cancer immunotherapy. Several aspects of these findings deserve further comment.

First, the role of RA in the induction of Tregs in the intestine and gut tolerance has been extensively studied (6, 7). However, its role in tumor-induced immune tolerance is poorly understood. TME has high levels of RA, and APCs are major producers of RA (8). Also, the signaling pathways that promote DCs to metabolize vitamin A into RA are not known. Our studies demonstrate that DCs in the TME acquire the ability to metabolize vitamin A via the activation of the β-catenin/TCF4 pathway, which programs them to promote regulatory T-cell responses. RA acts directly on CD4 cells inducing regulatory T-cell responses and suppresses T effector cell response. Deletion of β-catenin or TCF-4 in DCs resulted in significant decrease in Aldh1 expression, and these DCs were less potent in inducing Tregs in response to melanoma. Consistent with this observation, pharmacologic inhibition of Aldh1 activity significantly delayed tumor growth and enhanced the antitumor immunity. In addition to inducing Tregs, recent studies indicate that RA can also act directly on DCs imparting regulatory phenotype via induction of SOCS3 (26, 37).

Second, aberration in the β-catenin/TCF signaling pathway is associated with poor prognosis in many human tumors, but the focus of most research has been on the effects of this pathway on the tumor cells themselves (16, 38, 39). In models of gut tolerance, our previous studies have shown that β-catenin signaling in DCs programs them into a regulatory state and promotes regulatory T-cell responses (19, 32, 40); however, whether this pathway promotes immune suppression in tumors was not known. The present study clearly demonstrates that tumor-induced activation of the β-catenin/TCF4 pathway programs DCs into an immunosuppressive state and promotes immune tolerance. TME can also modulate DC function by regulating DC activation and maturation, by regulating the expression of costimulatory and coinhibitory molecules. Our study shows that deletion of β-catenin resulted in increased expression of costimulatory molecules and decreased expression of coinhibitory molecules. This phenotype is associated with DC in an immunogenic state, resulting in enhanced antitumor immunity. In addition to inducing Tregs, β-catenin activation in DCs can also affect cross priming of CD8 T-cell responses against tumors (18). Although β-catenin is known to suppress DC activation (7), it remains to be determined whether β-catenin directly regulates DC activation or does so indirectly via RA.

The receptor(s) on DCs or factor(s) in TME that activate β-catenin in DCs are currently not known. Emerging studies show that TME contains high levels of Wnt ligands (16, 38, 39), TGFβ (41), and TLR ligands (42). These molecules can also initiate signaling within DCs to activate the β-catenin pathway, to promote immune suppression. The role of these molecules if any in the activation of β-catenin pathway in DCs in TME is currently unknown. In addition, the downstream mediator of β-catenin signaling in DCs was not known. The present study shows that TCF4 is one of the key mediators of β-catenin signaling in DCs in TME. Upon activation, β-catenin is known to interact with other transcription factors such as PPARγ (10, 11), Foxo (12, 13), VDR (12, 14), and IRF3 (15), which are known regulators of immune responses, but additional studies will be required to determine the role of β-catenin in the regulation of these transcription factors and their target genes in response to antitumor immunity.

Other regulatory factors such as indoleamine 2,3-dioxygenase (IDO), IL10, and TGFβ, though not analyzed in present study, may play a role in tumor tolerance through the β-catenin/TCF pathway (3, 26, 40–42). The present study was focused on RA because blocking RA essentially recapitulated the effects of a conditional knockout of β-catenin in mice. Furthermore, exogenous addition of RA fully restored tumor growth and immune suppression in β-catenin conditional knockout mice. Thus, our data indicate that signaling initiated by the β-catenin/TCF4 pathway in DCs promotes both tumor growth and immune suppression.

In summary, our study reveals a novel mechanistic link between the β-catenin/TCF pathway in DCs, RA, and Tregs, by which tumors promote immune tolerance. The present data demonstrate that pharmacologic disruption of this pathway altered DC function, resulting in enhanced immune response against established tumors. These findings have important implications for tumor immunotherapy, suggesting that blocking the β-catenin pathway in DCs helps the goal of breaking tumor-induced immune suppression by enhancing antitumor immunity against established tumors.

Disclosure of Potential Conflicts of Interest

A.L. Mellor has ownership interest (including patents) and is a consultant/advisory board member for NewLink Genetics. No potential conflicts of interest were disclosed by the other authors.

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B. Manicassamy
Study supervision: S. Manicassamy
Other (reviewed the article and provided constructive feedback to the senior author): A.L. Mellor

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β-Catenin Promotes Regulatory T-cell Responses in Tumors by Inducing Vitamin A Metabolism in Dendritic Cells

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