

Wnt3a Neutralization Enhances T-cell Responses through Indirect Mechanisms and Restrains Tumor Growth



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Abstract

The Wnt/ β -catenin pathway regulates T-cell functions, including the repression of effector functions to the advantage of memory development via Tcf1. In a companion study, we demonstrate that, in human cancers, Wnt3a/ β -catenin signaling maintains tumor-infiltrating T cells in a partially exhausted status. Here, we have investigated the effects of Wnt3a neutralization *in vivo* in a mouse tumor model. Abundant Wnt3a was released, mostly by stromal cells, in the tumor microenvironment. We tested whether Wnt3a neutralization *in vivo* could rescue the effector capacity of tumor-infiltrating T cells, by administering an antibody to Wnt3a to tumor-bearing mice. This therapy restrained tumor growth and favored the expansion of tumor antigen-specific CD8⁺ effector memory T cells with increased expression of Tbet and IFN γ and reduced expression of Tcf1. However, the effect was not attributable to

the interruption of T-cell-intrinsic β -catenin signaling, because Wnt3a/ β -catenin activation correlated with enhanced, not reduced, T-cell effector functions both *ex vivo* and *in vitro*. Adoptively transferred CD8⁺ T cells, not directly exposed to the anti-Wnt3a antibody but infiltrating previously Wnt3a-neutralized tumors, also showed improved functions. The rescue of T-cell response was thus secondary to T-cell-extrinsic changes that likely involved dendritic cells. Indeed, tumor-derived Wnt3a strongly suppressed dendritic cell maturation *in vitro*, and anti-Wnt3a treatment rescued dendritic cell activities *in vivo*. Our results clarify the function of the Wnt3a/ β -catenin pathway in antitumor effector T cells and suggest that Wnt3a neutralization might be a promising immunotherapy for rescuing dendritic cell activities. *Cancer Immunol Res*; 6(8); 953–64. ©2018 AACR.

Introduction

Decades of research have consolidated the notion that growing tumors evolve a variety of mechanisms to evade immune surveillance. These mechanisms include escaping immune recognition, promoting regulatory cell expansion, coopting inhibitory signals, and secreting suppressive factors (1). Cancer immunotherapy has advanced with the development of immune checkpoint inhibitors, which block negative receptors in tumor antigen-specific T cells (2). However, to further improve immunotherapy, new targets and therapeutic protocols are needed. A plethora of soluble molecules is released in the tumor microenvironment (TME) that may mediate immune suppressive functions. Knowledge about whether their neutralization *in vivo* rescues antitumor immunity

and by what mechanisms may aid identification of immune checkpoints.

The Wnt family comprises 19 highly conserved secreted glycoproteins involved in diverse cellular processes such as proliferation, migration, commitment, and self-renewal. Wnts can play nonredundant roles in embryonic development, tissue homeostasis, and carcinogenesis (3). Through the canonical signaling pathway, Wnt promotes the stabilization and the accumulation of β -catenin, which translocates into the nucleus and acts as a transcriptional switch for the Tcf/Lef-dependent program. Aberrant Wnt signaling, due to somatic mutations in genes of the Wnt/ β -catenin pathway and leading to a constitutive activation, is an oncogenic driver in a variety of cancer types including colorectal cancer (4). Tumor cells also release Wnt proteins, particularly Wnt3a (5, 6), and Wnt secretion is required to sustain the tumor cell-intrinsic β -catenin pathway and its oncogenic potential despite mutations (6). Administration of a Wnt3a-neutralizing antibody restrains tumor proliferation *in vivo* in a mouse model of prostate cancer (7).

The Wnt/ β -catenin signaling pathway functions in the immune system in several ways, including in hematopoiesis, T and B cell development, peripheral T-cell activation, polarization and migration, and regulation of DC activities (8). Several pieces of evidence suggest that Wnt signaling, β -catenin stabilization, and Tcf1 activation suppress terminal effector differentiation of CD8⁺ T cells (9). In a companion paper (10), we show that T cells infiltrating human hepatocellular carcinoma or colorectal cancer are kept in an Eomes⁺Tbet⁻ semidysfunctional state through T-cell-intrinsic β -catenin activation. Here, we ask whether Wnt ligands released in the TME might exert tumor-promoting and

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tumor cell-extrinsic activities through the local inhibition of CTL effector functions. Indeed, we report here that Wnt3a neutralization *in vivo* in tumor-bearing mice restrains tumor growth and unleashes the expansion of tumor-specific CD8⁺ T cells with improved effector activities. This effect does not, however, derive from a direct benefit to tumor-infiltrating CD8⁺ T cells, but rather requires the intervention of other cells, such as dendritic cells (DC), which are kept in a tolerogenic state by Wnt3a in the TME.

Materials and Methods

Tumor cell line and mouse models

C57BL/6NCrI wild-type and CD45.1 (B6.SJL-Ptprc^aPepc^b/BoyCrI), 8- to 12-week-old male mice were purchased from Charles River Laboratories and maintained at the animal facility of Dipartimento di Scienze Anatomiche, Istologiche, Medico legali e dell'Apparato locomotore (SAIMLAL), "Sapienza" Università di Roma.

The MC38 colon adenocarcinoma cell line was kindly provided by Mario P. Colombo in 2013 (Fondazione IRCCS Istituto Nazionale Tumori, Milan, Italy). The 18.5 c-myc H-Ras^{V12} p53^{-/-} (hereafter "18.5") cell line of immortal transformed hepatoblasts, inducing liver-derived tumors with histological subtypes and markers characteristic of human hepatocellular carcinoma (HCC) when injected subcutaneously (s.c.; ref. 11), was developed and provided by Micol Ravà in 2017 (European Institute of Oncology, Milan, Italy). Tumor cell lines were cultured *in vitro* for 3 to 4 passages in complete Dulbecco's modified eagle medium (DMEM) with high glucose (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 2 mmol/L L-glutamine (Sigma-Aldrich), penicillin/streptomycin, nonessential aminoacids, sodium pyruvate (Euroclone), 50 μmol/L β-mercaptoethanol (Sigma-Aldrich), and 10 mmol/L Hepes (Aurogene) at 37°C in humidified 5% CO₂ atmosphere. The cell lines were not authenticated in the past year and were routinely tested for *Mycoplasma*. MC38 cells (5 × 10⁵) or 18.5 cells (8 × 10⁵) were injected s.c. into the middle flank and, after 2 weeks, mice were sacrificed and tumor volume (mm³) was calculated using the formula: (smaller diameter)² × larger diameter.

All procedures involving animals were approved by the Italian Ministry of Health (authorization #481/2015-PR), and performed in accordance with national law (Dlgs 26/2014) and the institutional animal care and use committee (Organismo preposto al Benessere Animale, SAIMLAL Department, "Sapienza" Università di Roma).

Dosage of Wnt3a release in cell line or explants by ELISA

Conditioned medium (CM) was obtained *ex vivo* from murine tissues, by incubating small tumor (tCM) or spleen (sCM) fragments in complete RPMI Dutch modified medium (1 μL medium per mg of tissue) for 6 hours at 37°C.

To obtain the supernatant (SN) from the MC38 and 18.5 lines, cells were seeded at a concentration of 1.25 × 10⁵ cells/well in a 12-well plate, and the SN was collected after a 48- to 72-hour culture (around 80% confluence) in complete DMEM medium supplemented with 10% FBS.

Wnt3a concentration in CM and SN was dosed using Wnt-3a ELISA Kit (MyBioSource) according to the manufacturer's instructions. Briefly, 100 μL per well of 1:2 or 1:3-diluted samples were added into the Wnt3a precoated microplate and incubated for

2 hours at 37°C. After treatment with biotin-conjugated Wnt3a-specific antibody and streptavidin-conjugated enzyme, a substrate solution was added and color intensity was measured with Multiskan FC (Thermo Scientific).

In vivo treatments with anti-Wnt3a or anti-PDL1 Abs

Mice with palpable (1–3 mm) tumor nodules received anti-Wnt3a or isotype control (R&D Systems) treatment, following two different schedules: (i) a single intraperitoneal injection of 100 μg at day 6; (ii) repeated (3, 4) intratumor injections of 25 μg, starting at days 5 to 6 after s.c. tumor transplantation, and repeating the treatment every 2 to 3 days. Mice were sacrificed at days 12 to 14 (24 hours after the last injection in the intratumor protocol), and spleen and tumor were collected for flow cytometry analysis. Two different anti-Wnt3a-neutralizing clones (217804 and 930769; R&D Systems) and respective isotype controls (Rat IgG2a and Rat IgG1; R&D Systems) were tested, giving similar results. The two clones were obtained using two different immunogens (*E. coli*-derived recombinant mouse Wnt-3a Ser36-Gln75, Trp219-Arg269 for clone 217804, and CHO-derived recombinant mouse Wnt-3a Ser19-Lys352 for clone 930769); both clones were not cross-reactive against mouse Wnt5A, Wnt1, Wnt4 and human Wnt3a aa1–355; cross-reactivity against mouse Wnt3 (sharing 86% sequence identity with Wnt3a) was not tested. In some experiments, tumor-bearing mice were treated with a single intraperitoneal injection of 100 μg anti-Wnt3a/isotype control at day 6 post-tumor inoculation, combined with repeated intraperitoneal injections of 250 μg anti-PDL1 (clone 10F.9G2, Bio X Cell) or isotype control (Rat IgG2b, Bio X Cell) at days 6, 8, 11, and 13.

Cell extraction from murine tissues

To extract tumor-infiltrating lymphocytes (TIL), murine tumors were mechanically dissociated in PBS 2% FBS onto a 70-μm cell strainer (Falcon) in a Petri dish, and a single cell suspension was obtained. To extract tumor-infiltrating dendritic cells (TIDC), tissues were digested with Collagenase IV (Sigma-Aldrich) for 15 minutes at 37°C before mechanical disaggregation. Then leukocytes were enriched through 40/80 Percoll (GE Healthcare) density gradient, collecting cells at the interface between 40% and 80% Percoll solutions. Splenocytes were obtained by mechanical dissociation, followed by incubation with ACK lysis solution (Gibco) for 4 minutes at 4°C for erythrocyte lysis.

Flow cytometry

For the analysis of TIL functions, cells were left untreated or restimulated 4 hours in the presence of Cell Stimulation Cocktail plus Protein Transport Inhibitors (eBioscience). After incubation with Fixable Viability Dye eFluor780 for 30 minutes at room temperature (RT), staining with APC-labeled H-2Kb dextramers (Immudex), complexed with p15E_{604–611} (KSPWFITL) MC38 immunodominant peptide (12, 13), was performed for 10 minutes at RT in the dark. Then, antibodies for surface markers were immediately added, and intracellular staining was performed after fixation and permeabilization for 30 minutes at 4°C, using Foxp3/Transcription Factor Staining Buffer Set according to the manufacturer's instructions (eBioscience). Combinations of the following antibodies were used: anti-CD8 Brilliant Violet 785 (BioLegend), anti-CD4 Brilliant Violet 605 (BioLegend), anti-CD44 Brilliant Violet 510 (BioLegend), anti-CD45.1

Brilliant Violet 605 (BioLegend), anti-PD1 PerCP-eFluor710 (eBioscience), anti-Tbet Brilliant Violet 421 or V450 (BD Biosciences), anti-IFN γ Brilliant Violet 711 (BioLegend), anti-Eomes PECy7 (eBioscience), anti-Foxp3 APC (clone FJK-16s, eBioscience), anti-IL17 PerCP-Cy5.5 (BioLegend), anti-Tcf1 Alexa Fluor 488 (Cell Signaling Technology), anti- β -catenin PE (R&D Systems), and anti-non-phospho active β -catenin (Ser33/37/Thr41; D13A1) PE (Cell Signaling Technology).

For BMDC and TIDC analysis, after Fixable Viability Dye eFluor780 labeling, cells were stained with the following antibodies: anti-CD11c PEDazzle594 (BioLegend), anti-CD252 (OX40L) PECy7 (BioLegend), anti-CD80 Brilliant Violet 421 (BioLegend), anti-I-A/I-E (MHCII) Brilliant Violet 711 (BioLegend).

To check PDL1 expression by the MC38 cell line, cells were incubated with biotinylated anti-B7H1/PDL1 (R&D Systems) for 30 minutes at RT, and staining with streptavidin PE (eBioscience) was performed for 15 minutes at RT.

To test Wnt3a production by the MC38 cell line, 5×10^5 cells per well were cultured in a 12-well plate in complete DMEM medium in the presence of Protein Transport Inhibitors (eBioscience) for 8 hours at 37°C. After incubation, cells were fixed/permeabilized for 20 minutes at 4°C with Cytotfix/Cytoperm solution according to the manufacturer's instructions (BD Biosciences), and stained with FITC anti-Wnt3a (clone #217804) or isotype control (RnD Systems).

Data were acquired on LSR Fortessa (Becton Dickinson) and analyzed with FlowJo software (TreeStar Inc., version 10.1r5).

Immunofluorescence

For immunofluorescence analysis, 4- μ m-thick tissue sections were deparaffinized and rehydrated. Subsequently, antigen unmasking was performed using the Novocastra Epitope Retrieval Solution pH 9 (Leica Biosystems), in PT Link (Dako) at 98°C for 30 minutes. Sections were brought to RT and washed in PBS. After protein blocking by a specific protein block (Novocastra, Leica Biosystems), samples were incubated overnight at 4°C with Rat Monoclonal anti-mouse CD3 (1/100 dilution, CD3-12 ab11089, Abcam). Primary Ab binding was revealed by Alexa Fluor 488 goat anti-rat IgG (H+L) from Invitrogen Molecular Probes. Subsequently, samples were incubated with Rabbit Polyclonal anti-mouse Wnt3a (1/500 dilution, Cat.#09-162 Lot# 2512436, EMD Millipore) for 1 hour at RT. Wnt3A Ab binding was revealed by Alexa Fluor 568 goat anti-rabbit IgG (H+L; Invitrogen Molecular Probes). Slides were counterstained with DAPI Nucleic Acid Stain (Invitrogen Molecular Probes). Sections were analyzed under the Axio Scope A1 optical microscope (Zeiss), and microphotographs were collected using the AxioCam 503 color digital camera (Zeiss).

In vitro TIL culture and Wnt3a neutralization assay

TILs (3×10^5 per well), obtained as previously described, were seeded in a U-bottomed 96-well plate and stimulated with soluble anti-CD3 (1 μ g/mL, eBioscience) in complete RPMI Dutch modified medium for 4 days at 37°C in the presence or absence of 1:4-diluted tCM, 400 ng/mL recombinant Wnt3a (R&D Systems), 20 μ g/mL anti-Wnt3a or isotype control (R&D Systems). Then, flow cytometry analysis was performed after treatment with Cell Stimulation Cocktail plus Protein Transport Inhibitors (eBioscience) in the last 4 hours of culture.

Adoptive CD8 T-cell transfer

Splenocytes were obtained from CD45.1 donor mice bearing 8–10 mm MC38 tumor nodules. CD8 T cells were magnetically purified from splenocytes using the CD8+ T cells isolation kit (Miltenyi Biotec), and labeled with CFSE by incubation for 15 minutes at 37°C with 10 μ mol/L CellTrace CFSE (Thermo Fisher). Around 5×10^6 CD8 T cells/mouse were intraperitoneally transferred into C57BL/6 (CD45.2) tumor-bearing host mice, at day 11 posttumor implantation, having previously received, at days 6 and 8, anti-Wnt3a or isotype intratumor treatment. At day 11, before T-cell transfer, the concentration of residual circulating Rat IgG in sera was 196–347 ng/mL (not shown), around 100-fold smaller than anti-Wnt3a concentrations tested *in vitro*. Mice were sacrificed at day 14 and tumors and spleens were collected and processed for analysis.

BMDC differentiation and *in vitro* Wnt3a neutralization assay

BMDC cells were obtained as previously described (14), with few modifications. Briefly, BM cells (7.8×10^6), collected through tibiae and femur flushing, were cultured in complete Iscove's modified Dulbecco medium (IMDM, Euroclone) supplemented with 10% FBS (Gibco), penicillin/streptomycin, 50 μ mol/L β -mercaptoethanol and 20 ng/mL recombinant mouse GM-CSF (PeproTech) in 100-mm Suspension Petri dish (Corning). After 3 days, fresh medium with 36 ng/mL GM-CSF was added, and at day 9 floating and poorly adherent cells were collected, after incubation with 3 mmol/L EDTA (EuroClone) for 2 minutes at RT. CD11c⁺ cells were enriched with CD11c MicroBeads Ultrapure (Miltenyi Biotec), and >90% pure BMDCs were used for *in vitro* assays.

BMDCs (2×10^5 /well) were cultured for 18 hours in U-bottomed 96-well plate in complete RPMI medium with or without 1 μ g/mL lipopolysaccharide (LPS, Sigma-Aldrich), 7 μ mol/L GSK-3 inhibitor (TWS119, TOCRIS Bioscience), 1:4-diluted tCM, anti-Wnt3a (20–50 μ g/mL), or isotype control (R&D Systems). Then, cells were analyzed by flow cytometry.

Statistical analysis

Statistical analysis was performed using Prism software (version 6.0, GraphPad). Two-tailed unpaired Student *t* test was used to analyze *in vitro* data and to compare *ex vivo* data. Every *in vitro* and *ex vivo* assay was performed in duplicate or triplicate when possible and was independently repeated at least twice. In all graphs, bars show means \pm SEM. In all tests, *P* < 0.05 was considered statistically significant.

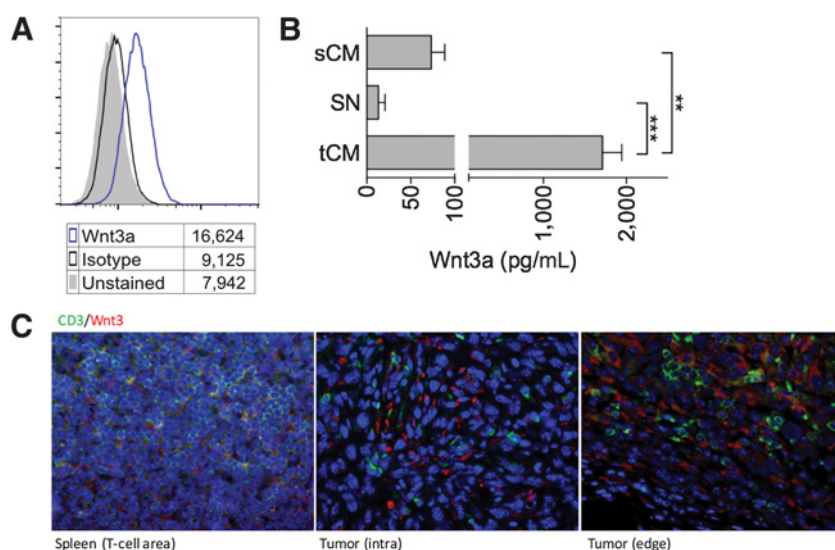
Results

Stromal cells in the TME produce Wnt3a *in vivo*

To investigate the roles of Wnt3a on the antitumor T-cell response, we took advantage of a well-established mouse tumor model, the subcutaneous implantation of the colon adenocarcinoma cell line MC38 (15). This tumor is particularly immunogenic, due to the presentation of a variety of mutated antigens (16), but also of epitopes derived from an endogenous retrovirus, such as the immunodominant peptide p15E_{604–611} derived from the envelope of murine leukemia virus (MuLV; refs. 12, 13).

First, we evaluated Wnt3a content in tumor cells and in tumor nodules. The MC38 cell line produced Wnt3a, as revealed by intracellular flow cytometry, and soluble Wnt3a could be detected through ELISA in the cell culture SN.

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**Figure 1.**

Wnt3a is produced by stromal cells in tumor nodules. **A**, Intracellular staining for Wnt3a in the MC38 cell line, after 8-hour incubation with protein transport inhibitors; numbers indicate median fluorescence intensity (MedFI). The experiment was repeated twice. **B**, Wnt3a concentrations, as assessed by ELISA, in the MC38 cell line 72-hour SN or in the CM obtained after 6-hour culture of spleen (sCM) or tumor (tCM) explants. Bars show means \pm SEM of 3 independent samples/type, each assayed in duplicate. **, $P < 0.01$; ***, $P < 0.005$, by Student *t* test, unpaired. **C**, Immunofluorescence of CD3 (green) and Wnt3a (red) in the indicated samples. One representative of spleens and tumors from 3 mice is shown.

However, Wnt3a concentration in the SN was significantly lower than in the CM of tumor explants (tCM; Fig. 1A and B), which contained Wnt3a amounts similar to what we find in human tumor explants (10). This result also suggests that tumor cells may boost their Wnt3a release *in vivo*, and/or that stromal cells in the TME may produce Wnt3a. Confocal immunofluorescence of tumor nodules (compared with spleen) revealed that Wnt3a was produced at the tumor site, more in peritumoral than in intratumor areas, and mostly by CD3⁻ cells with a myeloid cell morphology that were establishing close contacts with T cells (Fig. 1C).

Anti-Wnt3a restrains tumor growth and improves CD8⁺ T-cell responses

Our data suggest that Wnt3a from stromal cells in the TME may modulate the response of tumor-infiltrating T cells. To test this hypothesis, we treated tumor-bearing mice with Wnt3a-neutralizing antibodies and monitored tumor progression and antitumor CD8 response. In line with previous literature (7), the systemic (intraperitoneal) administration of a single dose of anti-Wnt3a-neutralizing antibody reduced tumor progression (Fig. 2A–C). However, when we performed repeated injections and local (intratumor) administration, we were able to achieve a stronger inhibition of tumor growth (Fig. 2D–F), suggesting that the blockade of microenvironmental Wnt3a may exert antitumor activity. We confirmed that this therapy was effective also against HCC-like nodules, containing Wnt3a in the TME, and derived from the subcutaneous injection of the 18.5 cell line of transformed hepatoblasts (Supplementary Fig. S1A–S1C).

To ascertain whether Wnt3a neutralization improved CD8⁺ T-cell responses *in vivo*, we profiled CD8⁺ T cells extracted from tumors (and spleens as control) 24 hours after the latest intratumor treatment. We measured the frequencies of CD8⁺ T cells specific for the MC38 immunodominant peptide (KSPWFITL, H-2Kb-restricted) through dextramer staining and flow cytometry analysis. We detected no differences in the spleen, but we found CD8⁺ T cells infiltrating Wnt3a-neutralized tumors were more enriched in tumor antigen-specific effector memory (CD44⁺) cells than were isotype-treated tumors (Fig. 3A and B). Both p15E_{604–611}-specific and nonspecific effector memory T cells

expressed more Tbet and IFN γ in tumors (and not in spleens) of anti-Wnt3a-treated mice, compared with controls, an effect more pronounced in the tumor-reactive compartment (Fig. 3C and D). This result suggests that Wnt3a neutralization promotes the expansion and polarization of a Tbet^{hi} and IFN γ -producing CD8⁺ T population, a cell type that has been reported to exert protective activities in tumors (17). This result also shows *in vivo* the efficacy of Wnt3a neutralization in rescuing Tbet expression, a result predicted by our *in vitro* results in human T cells (10).

Anti-Wnt3a treatment also affected the tumor-infiltrating CD4⁺ T-cell compartment, in terms of increased frequency and higher Tbet and IFN γ expression of CD44⁺ effector memory CD4⁺ T cells (Supplementary Fig. S2A–S2E), and in terms of reduced frequencies of Tregs but not Th17 cells (Supplementary Fig. S3A–S3D).

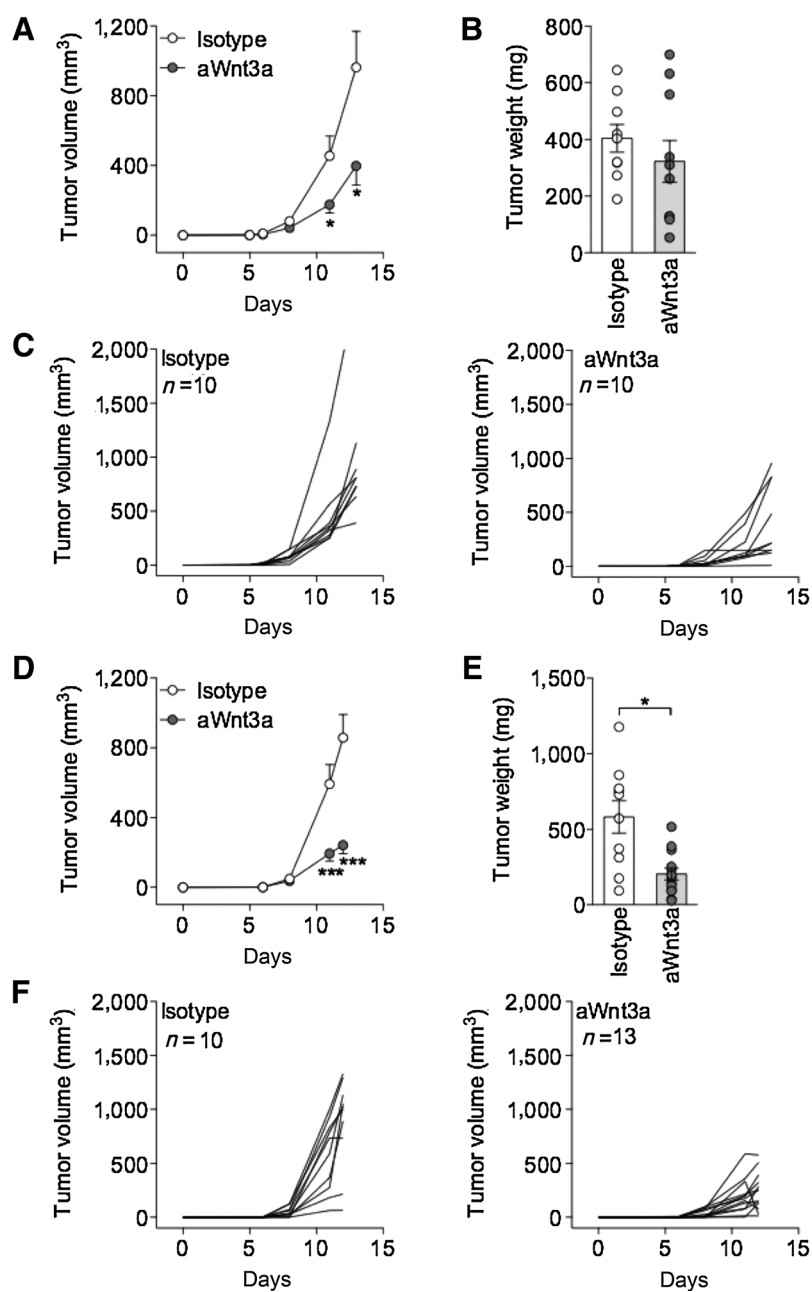
Overall, the therapy induced a significant increase in the CD8/CD4 ratio in the MC38 but not in the 18.5 model (Supplementary Fig. S4A). This difference may be ascribed to the lower CD8/CD4 ratio and immunogenicity of the 18.5 tumor at baseline. However, in 18.5-bearing mice, a tendency for increased CD4⁺ T-cell frequency and Tbet expression could be observed after anti-Wnt3a therapy (Supplementary Fig. S4B and S4C).

In several settings of chronic antigen exposure including cancer, Tbet^{hi} effector memory CD8⁺ T cells have been recognized as a heterogeneous population that can be further differentiated, on the basis of Eomes expression, into Tbet^{hi} Eomes^{lo} short-term memory precursors, which can give rise to fully differentiated Tbet^{hi} Eomes^{hi} effectors (17, 18). We found that anti-Wnt3a treatment increased the frequencies of both Tbet^{hi} Eomes^{lo} and Tbet^{hi} Eomes^{hi} subsets (the former to a higher extent), in both antigen-specific and nonspecific CD8⁺ effector memory T cells (Supplementary Fig. S5A and S5B), suggesting a control of microenvironmental Wnt3a on the general Tbet expression level in tumor-infiltrating CD8⁺ effectors.

T-cell exhaustion in chronically stimulated and dysfunctional (Tbet^{lo} Eomes^{hi}) CD8⁺ T cells is characterized by high expression of PD1 (19). The PD1/PDL1 axis is now recognized as a key player in tumor-associated CD8 dysfunctions (2). PDL1 blocking therapy can control tumor growth and recover CTL functions also in the MC38 model (20). PDL1 blockade synergizes with another therapeutic approach, i.e., MEK inhibition, which expands Tbet^{hi}

Figure 2.

Wnt3a neutralization *in vivo* restrains tumor growth. **A–C**, MC38 was s.c. injected in C57BL/6 mice, and at day 6 (when tumor was palpable) mice received a single i.p. inoculation of 100 μ g of anti-Wnt3a-neutralizing antibody (clone 217804) or isotype control (rat IgG2a). **A**, Means \pm SEM of tumor volume in each experimental group. *, $P < 0.05$, by the Student *t* test, unpaired, compared with isotype control. **B**, Means \pm SEM of tumor weight as measured at tumor excision. **C**, Curves representing tumor growth in single mice. Data shown in **A–C** are pooled from 2 independent experiments, comprising 10 mice per group. **D–F**, Mice bearing palpable MC38 nodules received repeated i.t. injections of 25 μ g of anti-Wnt3a antibody (clone 217804) or isotype control (rat IgG2a) at days 6, 8, and 11 after tumor transplantation. Similar results were obtained with another anti-Wnt3a clone (930769, rat IgG1). **D**, Means \pm SEM of tumor volume in each experimental group. ***, $P < 0.005$, by Student *t* test, unpaired, compared with isotype control. **E**, Means \pm SEM of tumor weight as measured at tumor excision. *, $P < 0.05$, by Student *t* test, unpaired. **F**, Curves of tumor growth in single mice. Data shown in **D–F** are pooled from 2 independent experiments, comprising 10–13 mice per group.



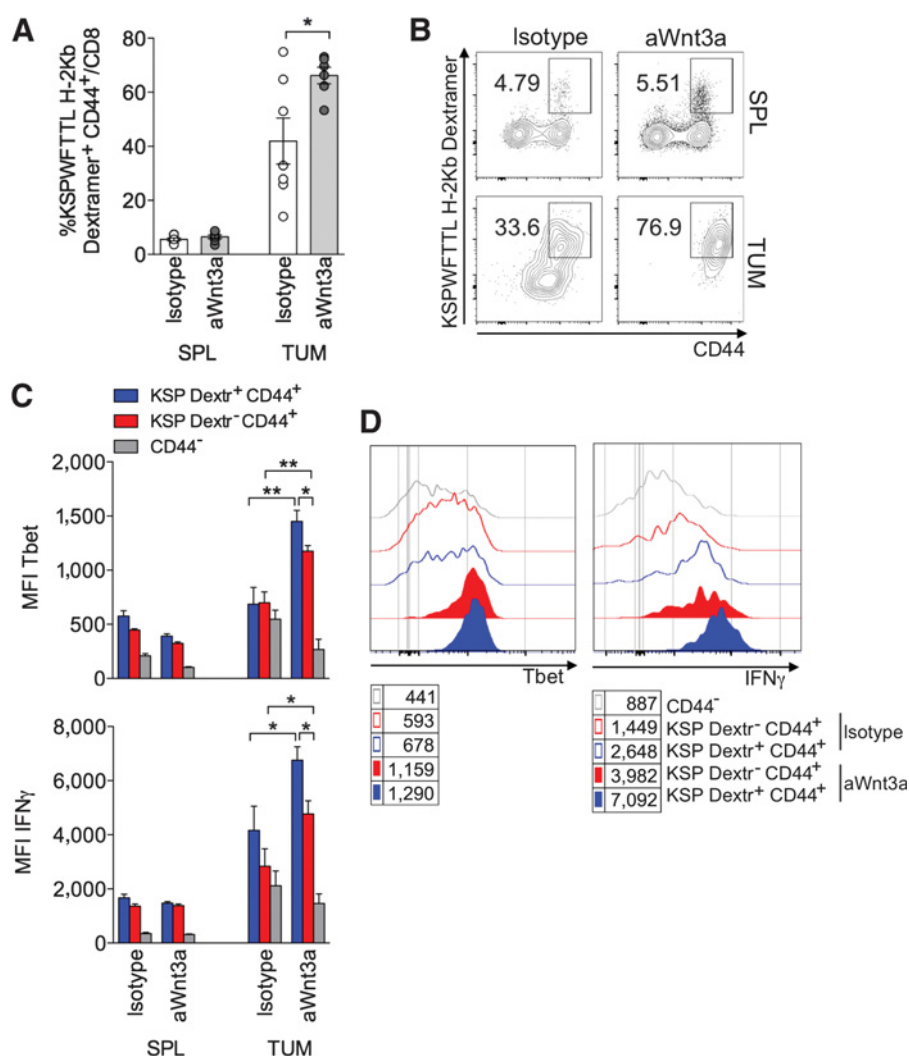
Eomes^{lo} and Tbet^{hi} Eomes^{hi} cells (17) similarly to our results with Wnt3a neutralization. Therefore, we tested whether anti-Wnt3a treatment affected PD1 expression in tumor-infiltrating CD8⁺ T cells, and whether it could be combined with anti-PDL1 administration. Confirming previous data (20), MC38 cells expressed PDL1 on the cell surface (Supplementary Fig. S6A). Tumor antigen-specific CD8⁺ T cells expressed PD1 at significantly higher levels than the nonspecific counterpart, in line with findings in human cancer (21). However, Wnt3a-neutralizing treatment did not change PD1 expression on any CD8⁺ T-cell subset (Supplementary Fig. S6B). The anti-Wnt3a/anti-PDL1 combination treatment did not alter tumor control over single administrations, suggesting lack of interaction between the two activities (Supplementary Fig. S6C).

Overall, these data indicate that Wnt3a neutralization *in vivo* restored a Tbet-driven effector program in tumor-infiltrating T cells, mimicking the effects of PD1/PDL1 checkpoint blockade.

CD8⁺ T-cell rescue does not need cell-intrinsic blockade of the Wnt/ β -catenin pathway

The transcription factor Tcf1, which is activated by the Wnt/ β -catenin signaling pathway, is required for long-term maintenance and reexpansion of chronically stimulated CD8⁺ T cells. Tcf1 also antagonizes the Tbet-driven effector program: Tcf1 and Tbet show opposite expression patterns in chronic infections (22), and Tcf1-deficient CD8⁺ T cells exhibit increased IFN γ production and Tbet expression (23). Based on these premises, we tested whether Wnt3a neutralization might inhibit the Tcf1/ β -catenin

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**Figure 3.**

Wnt3a neutralization *in vivo* increases antitumor CD8 response at the tumor site. C57BL/6 mice bearing palpable MC38 nodules received repeated i.t. injections of anti-Wnt3a antibody or isotype control at days 6, 8, and 11 after tumor inoculation. At day 12 (after 24 hours), mice were sacrificed and lymphocytes were isolated from spleens (SPL) and tumors (TUM) for flow cytometry analysis. Frequencies (A) and representative plots (B) of KSPWFTTL-H-2Kb dextramer⁺ CD44⁺ cells in gated live CD8⁺ T cells. Mean fluorescence intensity (MFI) in SPL and TUM (C) and representative histograms and MFI values in TUM (D) of Tbet and IFN γ expression in the indicated CD8⁺ subsets: CD44⁻ naive, KSPWFTTL-H-2Kb dextramer (abbreviated into KSP Dextr)⁺ CD44⁺ tumor antigen-specific effectors, or KSP Dextr⁻ CD44⁺ nonspecific effectors. Data shown in all panels are from one representative of 2 independent experiments. Bars represent means \pm SEM of $n = 6-7$ mice per group. *, $P < 0.05$; **, $P < 0.01$, by Student *t* test, unpaired.

pathway, thus unleashing Tbet expression and effector T-cell differentiation.

Less Tcf1 was detected in all CD8⁺ T-cell subsets, including p15E₆₀₄₋₆₁₁-specific cells, obtained from tumors than from spleens. This finding was consistent with Tcf1 downregulation occurring in activated T cells (23, 24). Anti-Wnt3a treatment induced a further reduction of Tcf1 selectively in tumor-reactive effectors (Fig. 4A and B). This result could not be attributed to a defect in the β -catenin pathway as the total β -catenin content, which was higher in p15E₆₀₄₋₆₁₁-specific CD8⁺ T cells at baseline, was significantly increased, and not decreased, following Wnt3a neutralization (Fig. 4C and D). When we estimated the amount of nonphosphorylated active β -catenin by flow cytometry, we found modest β -catenin activation, especially in CD44⁺ CD8⁺ T cells, in both spleens and tumors. This β -catenin activation was not affected by anti-Wnt3a treatment (Supplementary Fig. S7A and S7B).

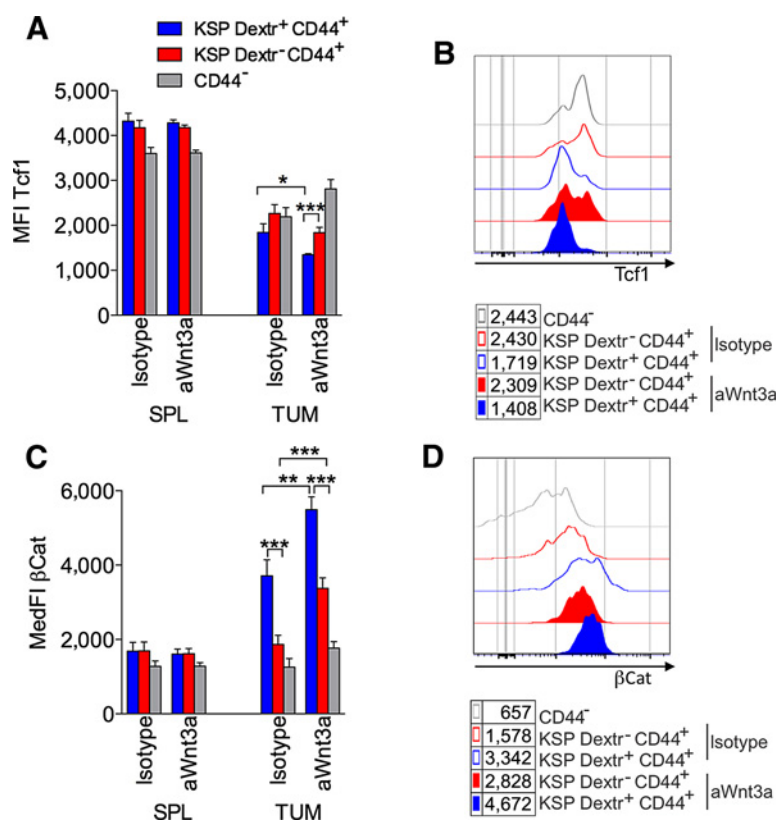
These data indicated that Wnt3a neutralization *in vivo* did not interrupt β -catenin signaling in CD8⁺ T cells but did increase total β -catenin levels, which led us to hypothesize that the treatment might improve *in vivo* CD8⁺ T-cell function indirectly, through inhibition of β -catenin signaling in other cells within the TME. To

test this idea, we first dissected the direct effects of Wnt3a stimulation and neutralization on CD8⁺ T cells *in vitro*. Recombinant Wnt3a (combined with anti-CD3) significantly increased both IFN γ and Tbet expression in CD8⁺ effector cells extracted from tumors (and not from spleens). On the other hand, Wnt3a neutralization significantly decreased Tbet levels in tumor-derived cells. tCM exposure downregulated Tbet/IFN γ expression, a suppressive effect not rescued by Wnt3a neutralization (Fig. 5A and B). These findings confirmed that Wnt3a signal might foster, rather than inhibit, the Tbet-driven program of tumor-infiltrating effector T cells, and that Wnt3a-neutralized tCM maintains its immunosuppressive activities against T cells *in vitro*.

To demonstrate the T-cell-extrinsic effects of Wnt3a neutralization *in vivo*, we performed an adoptive transfer experiment. CD8⁺ T cells were isolated from the spleens of CD45.1 tumor-bearing mice, CFSE-labeled, and injected into recipient CD45.2 mice bearing MC38 nodules previously treated with anti-Wnt3a or isotype control. In this setting, host cells experienced Wnt3a neutralization in the TME, while the donor-derived CD8⁺ T cells might only be exposed to minor amounts of antibody leaking out from the injection site. Thus, any improvement in adoptively transferred cell functions should be attributable to secondary,

Figure 4.

CD8⁺ T-cell rescue is associated with Tcf1 downregulation and β -catenin accumulation. MC38 tumor-bearing mice were treated with repeated intratumor injections of anti-Wnt3a or isotype antibodies, and flow cytometry was performed as detailed in the legend of Fig. 3. MFI in spleen (SPL) and tumor (TUM; **A**) and representative histograms and MFI values in tumor (**B**) of Tcf1 expression. MedFI in SPL and TUM (**C**) and representative histograms and MedFI values in TUM (**D**) of β -catenin expression. The following CD8⁺ subsets are shown: CD44⁻ naive, KSPWFTTL-H-2Kb dextramer (abbreviated into KSP Dextr)⁺ CD44⁺ tumor antigen-specific effectors, or KSP Dextr⁻ CD44⁺ nonspecific effectors. Data shown in all panels are from one representative of 2 independent experiments. Bars represent means \pm SEM of 7 mice per group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$, by Student *t* test, unpaired.



microenvironmental, effects. The results show a significant Tbet upregulation, and a not statistically significant IFN γ increase, in adoptively transferred CD8⁺ T cells (Fig. 5C and D), demonstrating that Wnt3a blockade can reshape the TME, thus indirectly promoting Tbet expression in CD8⁺ T cells.

Wnt3a neutralization rescues tumor-induced DC dysfunctions.

Crucial orchestrators of antitumor immunity, DCs are kept in immature and dysfunctional states in the TME (25). Several pieces of evidence indicate that Wnt/ β -catenin signal promotes tolerogenic activities in DCs (26–28). This pathway is active in DCs from tumor-bearing mice (29) and suppresses the activation of antitumor CD8⁺ T cells (29–31). Therefore, we sought to investigate whether TME-derived Wnt3a was responsible for the blockade of DC maturation. LPS-induced BMDC maturation was tested in the presence of a β -catenin stabilizer (the GSK3 inhibitor TWS119), tCM, anti-Wnt3a antibody or isotype control. Among the markers analyzed (MHCII, CD80, and OX40L), CD80 and particularly OX40L were more affected by the modulation of Wnt/ β -catenin pathway. Both β -catenin activation and tCM exposure suppressed the upregulation of CD80 and OX40L to a similar extent. Wnt3a neutralization abolished, in a dose-dependent fashion, the inhibitory effects of tCM on both markers (Fig. 6A and B). Finally, we tested whether Wnt3a neutralization affected TIDCs *in vivo* in MC38-bearing mice receiving repeated intratumor antibody injections. A not statistically significant increase in TIDC frequency and CD80 expression, and a significantly increased OX40L expression in TIDC, appeared in tumors of anti-Wnt3a-treated compared with isotype-treated mice (Fig. 7A–C).

In conclusion, our results reveal that Wnt/ β -catenin blockade in the TME exerts its antitumor therapeutic activities by unleashing

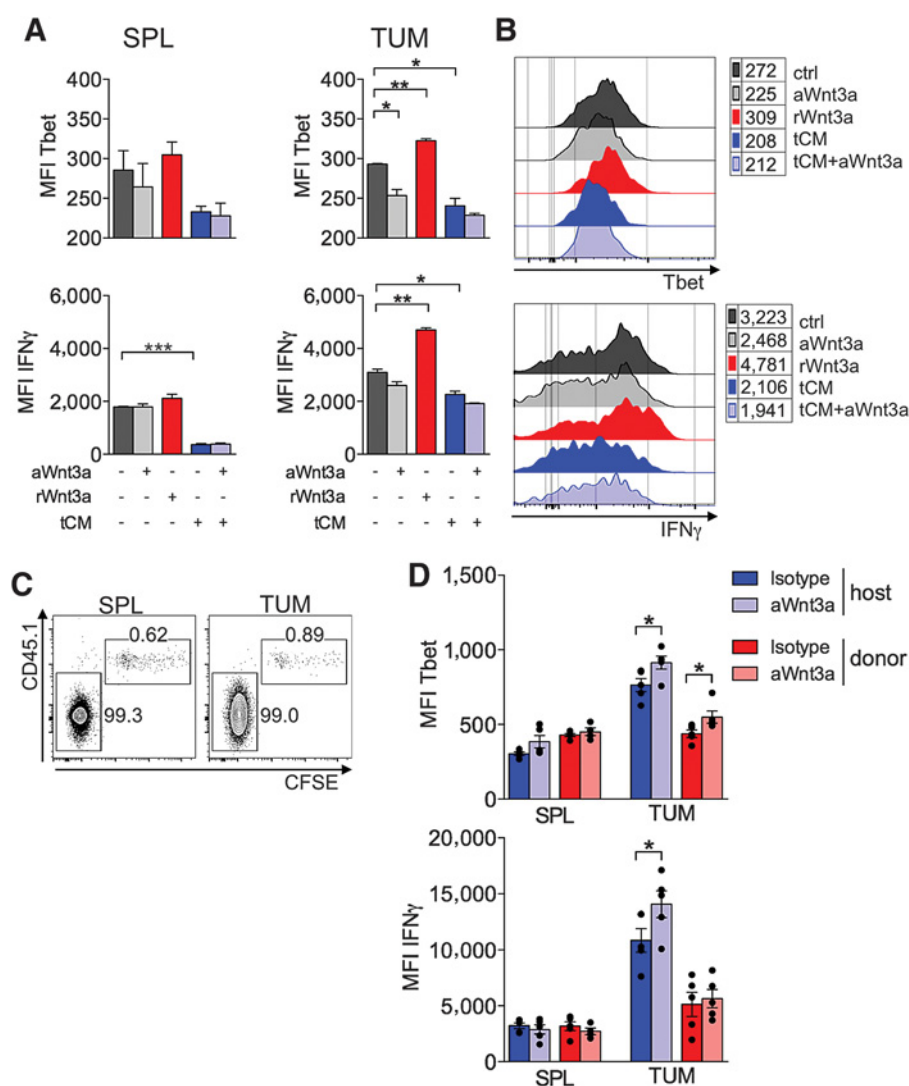
DC maturation, which in turns induces a secondary benefit with the development of a Tbet-driven effector program in CD8⁺ T cells.

Discussion

Activation of the Wnt/ β -catenin signal promotes carcinogenesis through the tumor-cell-intrinsic promotion of proliferative and invasive phenotypes. However, Wnt ligands are released in the TME, thus possibly initiating β -catenin activation in a variety of tumor-infiltrating immune cells. In a companion paper (10), we show that TME-derived Wnt3a activates the β -catenin pathway in human tumor-infiltrating T cells displaying a semiexhausted phenotype. Here, we show that Wnt3a neutralization *in vivo* in a mouse tumor model controls tumor growth and rescues the antitumor CD8⁺ T-cell functions likely through involvement of DCs with improved activities.

According to the literature, tumor cell-intrinsic as well as tumor cell-extrinsic mechanisms of Wnt/ β -catenin activation may contribute to arrange and modulate anticancer immunity. Because mutations in *Axin2* and *Sox9* have been reported in the MC38 cell line (32), we predict that the β -catenin signaling would be constitutively active in these cells. Therefore, we cannot exclude that, in our model, Wnt3a neutralization reshapes the immune landscape indirectly by impairing the tumor cell-intrinsic β -catenin pathway. However, our observation of greater Wnt3a release from tumor explants than from the tumor cell line, mostly by peritumoral stromal cells, suggests this pathway directly modulates tumor-infiltrating immune cells.

Our results did not provide evidence for a T-cell-intrinsic interruption of the Wnt/ β -catenin pathway in the protective effect

**Figure 5.**

The beneficial effects of Wnt3a blockade rely on T-cell-extrinsic mechanisms. **A–B**, Lymphocytes were isolated from spleen (SPL) and tumor (TUM) of MC38-bearing mice and stimulated 4 days with tCM (diluted 1:4), rWnt3a (400 ng/mL), or anti-Wnt3a (20 μ g/mL, or isotype in control conditions). Then, intracellular staining for Tbet and IFN γ was performed. MFI (**A**) and representative histograms and MFI values in TUM-derived cells (**B**) of Tbet and IFN γ expression, gated in live CD8⁺ CD44⁺ cells. Each condition was tested in duplicates. Data shown in **A** and **B** are from one representative of 2 independent experiments. Bars represent means \pm SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$, by Student t test, unpaired. **C–D**, C57BL/6 (CD45.2) MC38-bearing mice, previously treated with anti-Wnt3a or isotype, received adoptively transferred CFSE-labeled CD8⁺ T cells isolated from congenic CD45.1 tumor-bearing mice. After 3 days, flow cytometry analysis was performed. **C**, Representative plots showing CD45.1⁺ CFSE-labeled donor cells and CD45.1⁻ unlabeled host cells, gated in live CD8⁺ CD44⁺ cells. **D**, MFI of Tbet and IFN γ in the indicated cell subsets, experimental groups, and specimens. Data shown in **C–D** are from one representative of 2 independent experiments. Bars represent means \pm SEM of $n = 5$ mice per group. *, $P < 0.05$, by Student t test, unpaired.

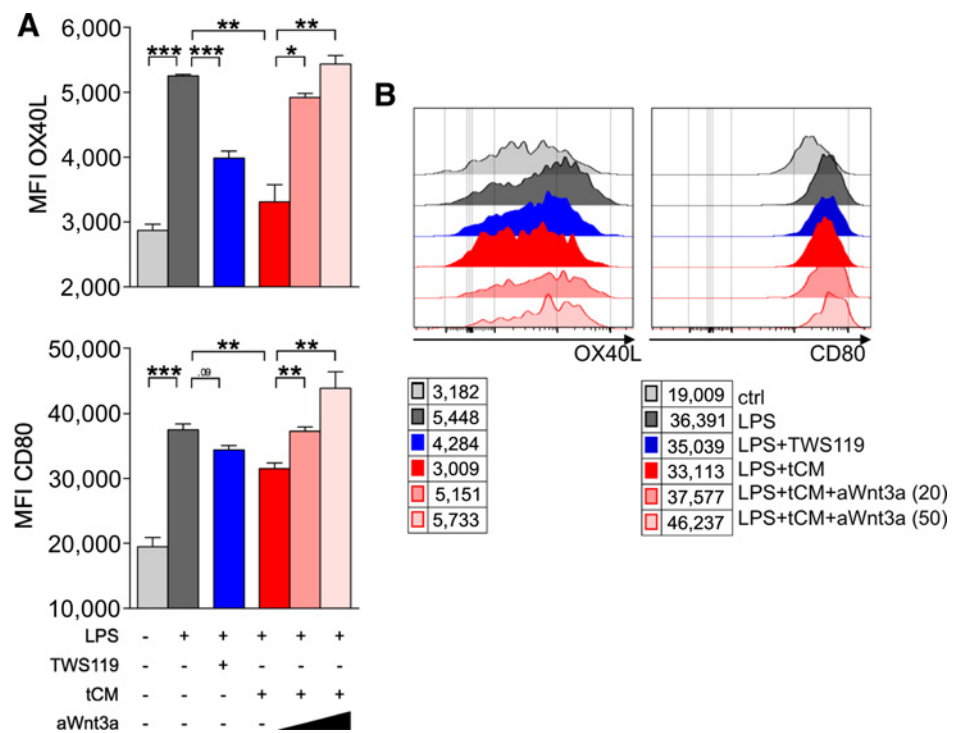
observed with anti-Wnt3a administration. First, following Wnt3a neutralization *in vivo*, β -catenin accumulated in T cells, more in antigen-specific than in nonspecific effector T cells. Higher β -catenin contents corresponded to expansion and improved effector functions of tumor-infiltrating CD8⁺ T cells. Second, Wnt3a stimulation *in vitro* enhanced IFN γ and Tbet levels, whereas Wnt3a neutralization inhibited Tbet expression in tumor-derived cells (a result suggesting that Wnt3a is released by cultured TIL). Third, anti-Wnt3a antibody failed to rescue the tumor-induced repression of IFN γ and Tbet, which may thus have been due to other immunosuppressive molecules present in the tCM, like TGF β (1). Fourth, adoptively transferred CD8⁺ T cells benefit from anti-Wnt3a treatment *in vivo* despite not being directly and locally exposed to the antibody. Fifth, we failed to detect any change in active β -catenin content in tumor-infiltrating T cells following Wnt3a neutralization *ex vivo*. Sixth, we observed Treg reduction in anti-Wnt3a-treated tumors, a result probably not attributable to a milder Wnt signaling in Tregs. Indeed, Wnt/ β -catenin activation restrains, rather than promotes, Treg development (33, 34). Overall, these results indicate that the target of Wnt3a blockade *in vivo* may not be the CD8⁺ T cells, and that the

Wnt/ β -catenin pathway has a beneficial effect on tumor-infiltrating effector memory cells. These results are in line with our observation that Wnt3a neutralization *in vitro* arrests human naive T-cell differentiation at a dysfunctional state, but does not affect the effector functions of already differentiated cells (10). Our data also suggest that the T-cell-intrinsic effects of Wnt3a neutralization to effector memory CD8⁺ T cells may be detrimental *in vivo* if not counterbalanced by indirect beneficial activities.

Reports have characterized the consequences of CD8⁺ T-cell-intrinsic activation of the Wnt/ β -catenin pathway and the involvement of Tcf1 (9, 23, 24, 35–38). Considerable evidence suggests an inverse relationship between Tcf1 activity and a Tbet-driven effector program. Tcf1-deficient T cells express more Tbet and a gene signature suggestive of improved effector functions (23, 37). Although Tcf1 represses the effector program, Tcf1 is retained and required during formation and recall of memory T-cell responses (9). A link has been demonstrated between Wnt/ β -catenin activation, Tcf1 binding to regulatory regions in the Eomes locus, and the Eomes-dependent memory program (37). Whether β -catenin stabilization affects T-cell activation and memory development remains unclear. β -Catenin-deficient T cells did not show defects

Figure 6.

Wnt3a neutralization reverses tumor-induced impairment in BMDC maturation. BMDCs were differentiated from C57BL/6 mice and >90% pure CD11c⁺ cells were cultured 18 hours with LPS (1 μg/mL), tCM (diluted 1:4), TWS119 (β-catenin stabilizer, 7 μmol/L), anti-Wnt3a, or isotype control (at two increasing concentrations, 20 and 50 μg/mL). MFI (A) and representative histograms and MFI values of OX40L and CD80 expression in B, gated in live CD11c⁺ MHCII⁺ cells. Each condition was tested in triplicates. Data shown in all panels are from one representative of 2 independent experiments. Bars represent means ± SEM. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.005, by Student *t* test, unpaired.



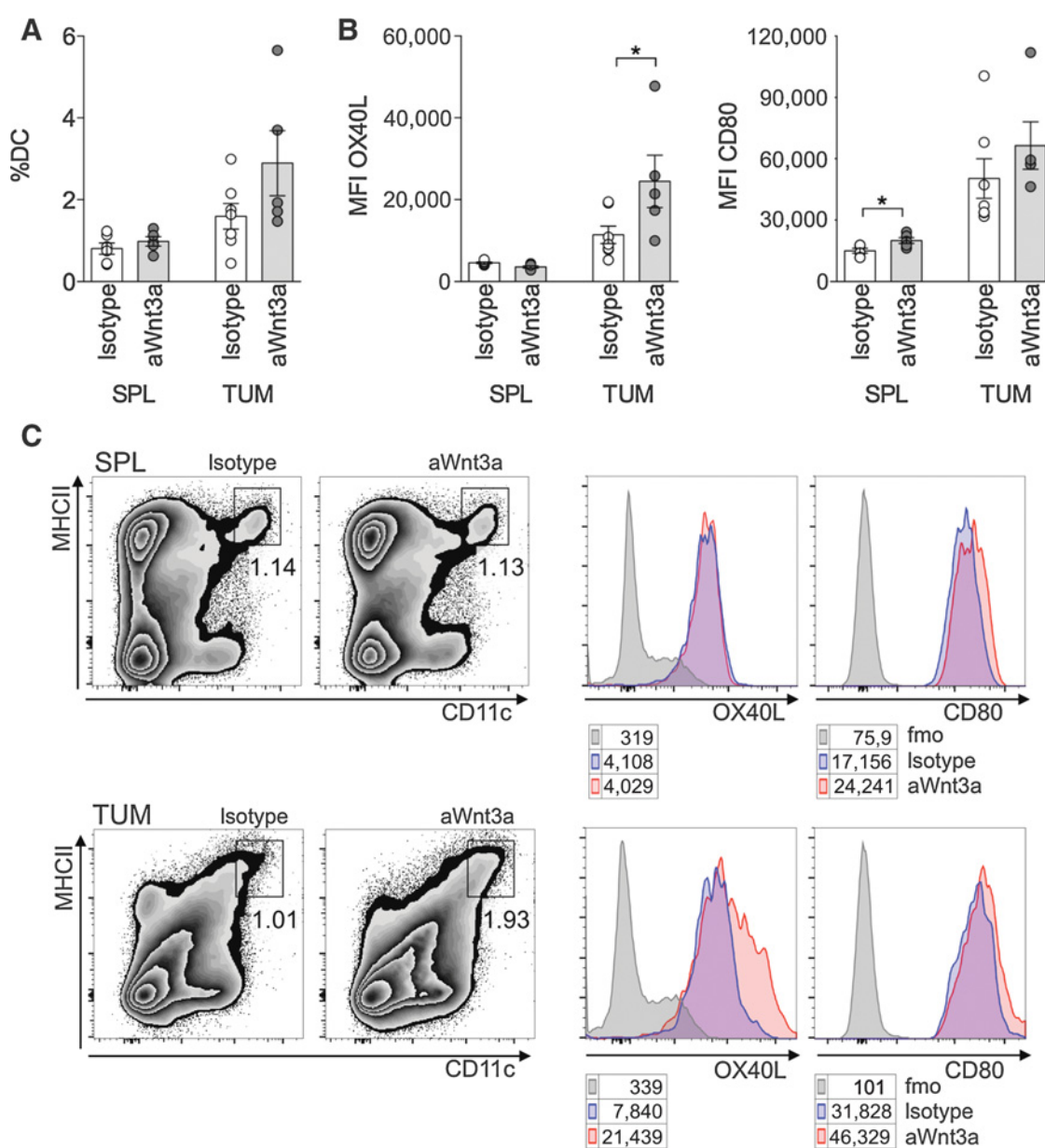
in effector or memory differentiation *in vivo* in one study (39), whereas another report demonstrated that nondegradable β-catenin-expressing T cells display poorer effector functions (40). Because Wnt-proficient systems were used in both studies, residual Wnt activity may account for the observed effects on T-cell activation in these systems. The consequences of Wnt/β-catenin triggering may differ depending on the responding cell (naïve or effector) and on concomitant stimuli (37). Our studies of Wnt3a and T cells in the human setting (10) speak in favor of divergent effects of Wnt3a in naïve versus effector T cells. We speculate that Tcf1 repression in antigen-experienced cells (36) may prevent Wnt3a-induced Eomes transcription, thus allowing development of Tbet-dependent effector function. This possibility may explain our results *in vivo* and *ex vivo* with tumor-infiltrating T cells. Indeed, the majority of CD8⁺ TILs are effector memory (CD44⁺ CD62L⁻) cells, and rescuing effector functions in these cells is a goal for active immunotherapy strategies. In line with this idea, we found that, *in vivo*, tumor-infiltrating effector memory CD8⁺ T cells expressed less Tcf1 compared with their splenic counterparts. Following anti-Wnt3a treatment, enhanced Tbet expression and IFNγ production corresponded to repressed Tcf1 levels selectively in tumor-specific effector CD8⁺ T cells, which preferentially experience antigen stimulation in the TME. We saw no change in Eomes expression in these cells. *In vitro*, combined TCR triggering and Wnt3a exposure, which represses Tcf1 (37), promoted Tbet and IFNγ increase only in tumor- but not spleen-derived CD8⁺ CD44⁺ T cells, respectively, containing mostly effector memory and central memory T cells. These data indicate that Tcf1 repression may favor development of a Tbet-driven effector memory program in T cells, thus exerting protective antitumor abilities.

Our conclusions are in apparent discrepancy with those of Gattinoni and colleagues, who showed a superior antitumor efficacy of CD8⁺ memory stem cells with enforced Tcf1 persis-

tence when adoptively transferred (24). However, tumor-infiltrating CD8⁺ T cells mostly comprise effector memory T cells at late and exhausted differentiation stages. These endogenous cells are the targets and mediators of checkpoint blockade-based therapies (41). Results from mouse models demonstrate that the rescue of CD8⁺ T-cell effector functions, such as Tbet-driven IFNγ release, is key to success (17, 41, 42). In human cancer, we demonstrated that IFNγ production can be recovered *ex vivo* selectively in Tbet^{hi} tumor-infiltrating CD8⁺ T cells (43). In line with previous findings in other mouse models (41), we found that Wnt3a neutralization exerted immunological effects mostly at the tumor site and not in the spleen, highlighting the relevance of tumor-infiltrating effector T-cell reactivation, rather than naïve or central memory T-cell expansion. In conclusion, we propose that, although Tcf1-Eomes enforcement may facilitate memory T-cell generation for adoptive cell transfer, Tcf1 repression and Tbet upregulation may reactivate endogenous effector T cells. We were able to drive Tcf1 decrease selectively in tumor-specific CD8⁺ T cells through Wnt3a neutralization *in vivo*. However, the mechanism probably was not through T-cell-intrinsic Wnt3a/β-catenin signaling. More likely, engagement of the TCR and/or a costimulatory receptor was stronger at the TME site, as suggested by *in vitro* data (37).

The most likely candidate for the primary target of anti-Wnt3a treatment in our tumor model is the DC. Wnt3a/β-catenin activation in DCs promotes a shift from proinflammatory to anti-inflammatory cytokines and supports their tolerogenic activities in a mouse model of autoimmunity (26–28). β-Catenin is active in DCs from tumor-bearing mice (29), and DCs with constitutive β-catenin stabilization cannot efficiently cross-prime effector T cells and maintain tumor immune surveillance (29, 30). Conversely, DC-restricted Wnt receptor blockade reduces tumor growth, augments antigen uptake by DCs and cross-priming of CD8⁺ T cells, and thus leads to improved effector functions in

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**Figure 7.**

Wnt3a neutralization rescues TIDC frequency and expression of activation markers *in vivo*. MC38 tumor-bearing mice were treated with repeated intratumor injections of anti-Wnt3a or isotype antibodies, and flow cytometry was performed. **A**, Frequency of MHCII^{high} CD11c⁺ DCs among total live leukocytes. **B**, MFI of OX40L and CD80 expression, gated in live MHCII^{high} CD11c⁺ cells. Bars represent means ± SEM of $n = 5-7$ mice per group. *, $P < 0.05$, by Student *t* test, unpaired. **C**, Representative plots and histograms showing MHCII^{high} CD11c⁺ cell frequency, OX40L and CD80 expression in the indicated samples; numbers indicate the MFI; fmo, fluorescence-minus-one control. Data shown in all panels are from one representative of 2 independent experiments.

CD8⁺ T cells (31). These results mirror our results *in vivo* with anti-Wnt3a administration in the MC38 model. By exposing BMDCs to tCM, we could recapitulate *in vitro* aspects of the tumor-induced suppression of DC maturation. In this setting, we could confirm that β -catenin stabilization inhibited BMDC maturation, and that the Wnt3a released in the TME was responsible for the immunosuppressive effect. We confirmed that Wnt3a neutralization also rescued TIDC functions *in vivo*. Among the myeloid cells that express Wnt3a *in vivo* at the tumor sites, both macrophages and

DCs can be included based on evidence in the literature (8). Autocrine/paracrine Wnt3a may sustain β -catenin signal and establish a tolerogenic program in tumor-infiltrating DCs, which could be interrupted by Wnt3a neutralization.

OX40L is among the molecules upregulated on both BMDCs and TIDCs following Wnt3a blockade. OX40L is expressed in murine BMDCs (44). Its receptor OX40 has costimulatory roles in both CD4⁺ and CD8⁺ T cells. Reverse signaling through OX40L may regulate DC activities (reviewed in ref. 45). Future studies will

ascertain whether Wnt3a neutralization synergizes with therapeutic agents addressed at OX40/OX40L signal enforcement.

In conclusion, our report in mouse models, together with our companion study in T cells extracted from human cancers, demonstrates that the T-cell-intrinsic Wnt/ β -catenin signaling may sustain the effector program of tumor-infiltrating CD8⁺ T cells. Other cells in the TME, including DCs, may benefit from Wnt3a neutralization *in vivo* and thus rescue immune surveillance. Therefore, Wnt3a neutralization, delivered ideally through DC-targeted approaches, may reshape the tolerogenic TME into a more immunogenic milieu.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): I. Pacella, I. Cammarata, A. Gulino, C. Tripodo
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Tripodo, V. Barnaba, S. Piconese

Writing, review, and/or revision of the manuscript: I. Pacella, I. Cammarata, V. Barnaba, S. Piconese

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): I. Pacella, I. Cammarata, C. Focaccetti, S. Miacci, M. Ravà

Study supervision: V. Barnaba, S. Piconese

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Wnt3a Neutralization Enhances T-cell Responses through Indirect Mechanisms and Restrains Tumor Growth

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