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Immune Suppression and Resistance Mediated by Constitutive Activation of Wnt/β-Catenin Signaling in Human Melanoma Cells

Tonomori Yaguchi,* Yasufumi Goto,† Kenji Kido,‡ Hiroshi Mochimaru,*‡ Toshiharu Sakurai,* Nobuo Tsukamoto,* Chie Kudo-Saito,* Tomonobu Fujita,* Hidetoshi Sumimoto,* and Yutaka Kawakami*

Cancer-induced immunosuppression is a major problem reducing antitumor effects of immunotherapies, but its molecular mechanism has not been well understood. We evaluated immunosuppressive roles of activated Wnt/β-catenin pathways in human melanoma for dendritic cells (DCs) and CTLs. IL-10 expression was associated with β-catenin accumulation in human melanoma cell lines and tissues and was induced by direct β-catenin/TCF binding to the IL-10 promoter. Culture supernatants from β-catenin–accumulated melanoma have activities to impair DC maturation and to induce possible regulatory DCs. Those immunosuppressive culture supernatant activities were reduced by knocking down β-catenin in melanoma cells, partly owing to downregulation of IL-10. Murine splenic and tumor-infiltrating DCs obtained from nude mice implanted with human mutant β-catenin–overexpressed melanoma cells had less ability to activate T cells than did DCs from mice with control melanoma cells, showing in vivo suppression of DCs by activated Wnt/β-catenin signaling in human melanoma. This in vivo DC suppression was restored by the administration of a β-catenin inhibitor, PKF115-584. β-catenin–overexpressed melanoma inhibited IFN-γ production by melanoma-specific CTLs in an IL-10–independent manner and is more resistant to CTL lysis in vitro and in vivo. These results indicate that Wnt/β-catenin pathways in human melanoma may be involved in immunosuppression and immunoresistance in both induction and effector phases of antitumor immunoresponses partly through IL-10 production, and they may be attractive targets for restoring immunocompetence in patients with Wnt/β-catenin–activated melanoma. *The Journal of Immunology, 2012, 189: 2110–2117.

Immunothe rapies often result in insufficient antitumor effects. For example, active immunization with MART-1 melanoma Ag peptides showed only a weak antitumor effect (1), but the recent adoptive T cell immunotherapy following lymphodepletive treatment using anti–MART-1 T cells resulted in a dramatic tumor reduction (2). These clinical trials indicated that one of the factors associated with better antitumor effects was elimination of immunosuppressive factors, including regulatory T cells (Tregs) and regulatory dendritic cells (DCregs) (3). Therefore, further understanding of the mechanisms involved in cancer cell–induced immunosuppression is essential for the future development of more effective immunotherapies.

Various mechanisms leading to the immunosuppression in cancer patients have been reported (4). However, it is not at present clear which are major suppressive factors and whether depletion of a single immunosuppressive factor among multiple suppressive mechanisms is sufficient to improve immunocompetence of cancer patients. In the cancer patients, cancer cells trigger production of multiple immunosuppressive molecules such as TGF-β and IL-10 and induce immunosuppressive cells such as Tregs and DCregs through interactions with other surrounding cells, including various immune cells and stromal cells (4). However, how cancer cells trigger the immunosuppressive cascades is still not completely understood. We have previously reported that the constitutively active BRAFV600E mutation and activated STAT3, which are frequently detected in human melanoma, lead to the production of multiple immunosuppressive cytokines (5), indicating that inhibitors for these signaling pathways may be useful for inhibition of multiple immunosuppressive factors to improve immunocompetence of the melanoma patients. In particular, possible use of STAT3 inhibitors for restoring immunocompetence in murine tumor models has been demonstrated (6). Therefore, further understanding of molecular mechanisms for human cancer cell triggering of immunosuppressive cascades is important to develop methods to restore immunocompetence of patients for improvements of immunotherapies.

β-catenin acts as a transcriptional coactivator by interacting with the TCF/LEF to regulate target genes in the nucleus. Mutations in
the β-catenin or APCs, a component of a complex associated with β-catenin degradation, which can induce β-catenin stabilization and nucleus translocation, have been identified in various human malignancies, including melanoma. The resulting upregulation of β-catenin has been shown to produce aberrant transactivation of downstream proto-oncogenes such as cyclin D1 and c-Myc (7, 8). Although mutations of β-catenin are relatively rare in melanoma tissues, nuclear and/or cytoplasmic accumulation of β-catenin was seen in one third of melanoma specimens by unknown mechanisms (11), and it might contribute to various malignant phenotypes (12). Interestingly, it has been recently suggested from melanoma gene microarray data that an activated Wnt/β-catenin signal in cancer microenvironment is correlated with lack of an immune infiltrate (13).

In this study, we demonstrated that the Wnt/β-catenin signaling pathway in human melanoma was involved in the production of an immunosuppressive cytokine, IL-10, and possibly other immunosuppressive molecules, which resulted in immunosuppression through impairment of DCs and effector T cells, indicating that Wnt/β-catenin signaling might be an attractive target for restoration of immunocompetence of melanoma patients.

Materials and Methods

Cells, culture supernatants, tissue samples, and regents

Culture media as follows: RPMI 1640 with 10% FBS (melanoma), DMEM with 10% FBS (HeLa and HEK293T), and human melanocyte growth supplement with medium 154S (Kurabo, Osaka, Japan) (melanocytes). Tumor-infiltrated lymphocytes (TILs), TIL397 and TIL1362, were generated as previously reported (14). Melanoma culture supernatants were obtained by culturing the cells at 1 × 10^6 cells/1.5 ml/well in a six-well plate for 24 h. Eighteen primary cutaneous melanomas and 18 metastases obtained with informed consent from 31 patients followed at Shinshu University Hospital were included in the immunohistochemical studies. All tissue samples were fixed in neutral buffered formalin and embedded in paraffin. Immunohistochemistry was done as previously described (15) using anti-human β-catenin Ab (DakoCytomation) and anti-human IL-10 Ab (R&D Systems). PKF115-584, acquired from Novartis (Basel, Switzerland), was resuspended in DMSO (10 mM stock solution).

Evaluation of β-catenin and IL-10 expression

DNA microarray analyses were performed as previously described (16). IL-10 mRNA expression was determined by real-time PCR using a TaqMan probe (Applied Biosystems). The expression was normalized to the level of GAPDH. IL-10 protein was measured by ELISA (BD Biosciences Pharmingen). Abs used in Western blotting are anti-GAPDH (Santa Cruz Biotechnology), anti-β-catenin (Sigma-Aldrich), and anti-β-actin (Cell Signaling Technology).

Reporter assay

Human β-catenin cDNA and genomic DNA of human IL-10 promoter region (GenBank accession no. AF295024; http://www.ncbi.nlm.nih.gov/nuccore/AF295024) were extracted from 888mel and subcloned into pME-18S and pGL4.10 vectors (Promega), respectively. Site-directed mutant was generated as previously described (17) using mutagenic primers, 5′-ccacctggagccccgagtggattgaga-3′ in the reporter assays, pME-18S-β-catenin cDNA, reporter plasmids, and phRL-SV40 were introduced into cells using Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, the cells were assayed using a PicaGene Dual SeaPanxy luminescence kit (Toyo Ink, Tokyo, Japan). Luciferase values were normalized to constitutive Renilla luciferase generated by cotransfected phRL-SV40 plasmid (Promega).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were conducted using an EZ ChIP kit (Upstate Biotechnology) according to the manufacturer’s protocol. Samples were subjected to immunoprecipitation using goat polyclonal anti-β-catenin Ab (Santa Cruz Biotechnology) and mouse monoclonal anti-TCF4 Ab (Cell Signaling Technology) and analyzed by 35-cycle PCR. The primers used for PCR were as follows: forward, 5′-AGTCTTGGGTATTCATCCCAGG-3′ and reverse, 5′-GAGCCTCTCCTTCATACCTC-3′ (IL-10 promoter); and forward, 5′-CTCTAAGTTGAGCAAGAATCACTT-3′ and reverse, 5′-TGGGACACCGGAGTACCTC-3′ (HSP70 promoter).

Knockdown or overexpression of β-catenin

The oligonucleotide small interfering RNA (siRNA) target sequence of human β-catenin was 5′-CCTGCGGATGAGTACAACAGAGGA-3′ (Invitrogen). Fifty to 100 nM siRNA was introduced into melanoma cells using Lipofectamine 2000. After 72 h incubation, cells were replated for assays. HIV vectors for short hairpin RNA (shRNA) expression or the mutated β-catenin expression were prepared as previously described (16). The shRNA target sequences were as follows: 5′-GCAACAGTCTTACCTGGAC-3′ (β-catenin) and 5′-GTGGCGCTCTGCTGTCAAC-3′ (firefly luciferase [GL3B], control shRNA). Melanoma cells overexpressing β-catenin were established by puromycin selection and their cell proliferations were evaluated using Premix WST-1 solutions (Tokara Bio, Shiga, Japan) 3 d after reseding the cells.

DC differentiation

PBMCs were obtained from healthy donors with informed consent. Human monocyte-derived DCs (Mo-DCs) were generated as previously reported (5). Briefly, human CD14+ monocytes were cultured with GM-CSF and IL-4 to differentiate without human IL-10 (8 ng/ml) or 20% (v/v) culture supernatants from 624mel transfected with β-catenin siRNA or scramble siRNA, and with 1 μg/ml IL-10–neutralizing Ab (R&D Systems) or isotype-matched control Ig. On day 5, DCs were washed, reseeded, and stimulated with 1 μg/ml LPS. On day 6, the culture supernatants were collected to measure IL-10 and IL-12 by ELISA (BD Biosciences Pharmingen), and DCs were used in MLR and flow cytometry analysis. Abs used for FACS staining were as follows: human CD1a, CD40, CD80, CD83, CD86, HLA-DR (Beckman Coulter), programmed death-ligand (PD-L1, PD-L2) (ebiScience), Ig-like transcript (ILT)-3, and ILT-4 (R&D Systems).

MLR and Treg suppression assays

Irradiated DCs (1.6 × 10^6) and allogeneic CD3+ T cells (1.6 × 10^5) were cocultured in 96-well plates. On day 3, IFN-γ was measured by ELISA (M700a and M701b; Endogen). On day 5, the induction of CD4+CD25+ FOXP3+ Tregs was analyzed by flow cytometry using anti-human CD4, CD25 (BD Biosciences Pharmingen), and FOXP3 Abs (ebiScience). CD4+CD25+ Tregs were isolated using MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany) and cocultured for another 6 d with autologous CD4+ responder cells (5 × 10^5 cells/well) with CD2, CD3, and CD28 Ab stimulus (Treg suppression inspector; Miltenyi Biotec) at different ratios. As controls, Tregs alone or responder cells alone without any stimulation were included. Proliferation of T cells was determined using BrdU incorporation (cell proliferation ELISA, BrdU kit; Roche). BrdU was added for 24 h after the 6-d culture.

In vivo functional analysis of splenic DCs

Mouse care and all experimental procedures were performed under pathogen-free conditions in accordance with established guidance of Keio University for animal experiments. The 397mel-β-catenin or -mock (5 × 10^5) were s.c. injected into the flank of nude mice (BALB/c, nu/nu). In PKF115-584 treatment models, 5 × 10^4 928mel was s.c. injected into SCID mice (C.B-17/Icr-scid). Treatment began 2–3 wk later. Control mice were measured by ELISA (BD Biosciences Pharmingen), and DCs were used in MLR and flow cytometry analysis. Abs used for FACS staining were as follows: human CD1a, CD40, CD80, CD83, CD86, HLA-DR (Beckman Coulter), programmed death-ligand (PD-L1, PD-L2) (ebiScience), Ig-like transcript (ILT)-3, and ILT-4 (R&D Systems).

Functional assay of human melanoma-specific CTLs

An IFN-γ release assay was performed as previously described (18). Briefly, 5 × 10^4 TIL397 and 1 × 10^5 melanoma cells were cocultured with 1 μg/ml IL-10–neutralizing Ab or an isotype-matched control Ig in a 96-well plate for 24 h. For [35]Cr-release assay, Na[35]CrO_4-labeled target
melanoma cells were cocultured with TIL1362 at various E:T ratios for 6 h and the supernatant radioactivity was measured. TIL1362 and 397mel express HLA-A01/25, 624mel expresses HLA-A02/03. In Winn assay, 4 × 10^5 tumor cells mixed with 8 × 10^5 TILs were s.c. injected into the flanks of NOG mice. As a control, tumor cells alone were inoculated. Mice were observed on a daily basis, and tumor volumes were calculated.

Results

High IL-10 expression in human melanoma with the activated β-catenin signaling pathway

To investigate the role of Wnt/β-catenin signal activation in immunobiology of human melanoma, we performed a systematic gene expression analysis using DNA chips on human melanoma cell lines with or without activated Wnt/β-catenin signaling. From the eight melanoma cell lines evaluated, we found that IL-10 was expressed in 5 of 16 melanoma cell lines by quantitative RT-PCR and ELISA (Fig. 1B). Four of the five IL-10–producing melanoma cell lines (501mel, 624mel, 888mel, and 928mel) were found to have the abnormal accumulation of β-catenin protein owing to either β-catenin stabilizing mutations or APC defects (Fig. 1B) (10), and we also found that high IL-10 production was observed only in β-catenin–accumulated melanoma (Table I; p = 0.0027, by Fisher exact test). Furthermore, in human melanoma tissue samples, IL-10 production was more frequently observed in melanoma tissues, which showed cytoplasmic and nuclear β-catenin accumulation by immunohistochemical analysis (Fig. 1C, Table I; p = 0.00079, by Fisher exact test). These observations indicate that activation of the Wnt/β-catenin signaling pathway may be involved in the high IL-10 production of human melanoma.

β-catenin directly promotes IL-10 expression through binding a β-catenin/TCF binding element in the IL-10 promoter

To determine whether constitutive activation of the Wnt/β-catenin signaling pathway regulates IL-10 expression in melanoma cells, we disrupted this pathway using β-catenin–specific RNA interference (RNAi). RNAi specific for two different target sequences in the human β-catenin gene were introduced into the two melanoma cell lines harboring mutated β-catenin, 624mel and 888mel, by using either a lentiviral vector encoding shRNA or oligonucleotide siRNA. Both of the anti–β-catenin RNAi significantly downregulated the IL-10 expression without affecting production of other cytokines, such as TGF-β and vascular endothelial growth factor, and cellular proliferation (Fig. 2A and data not shown). Next, β-cateninS37F, which is resistant to degradation, was overexpressed by lentiviral-mediated stable transfection in non–IL-10–producing melanoma cell lines without β-catenin accumulation, that is, 397mel and 938mel. Overexpression of β-catenin resulted in the production of a high amount of IL-10 by these melanoma cell lines (Fig. 2B). Therefore, activated Wnt/β-catenin signaling is associated with the IL-10 production by human melanoma.

There are seven possible β-catenin/TCF binding elements (TBEs: T/A T/A CAA T/A G) (19) in the human IL-10 promoter (Table II). Reporter assays where β-cateninS37F was transfected into HeLa cells along with a series of deletion constructs of IL-10 promoter reporter vectors showed the presence of β-catenin/TCF

![FIGURE 1. IL-10 production by β-catenin–accumulated human melanoma cells. (A) Dendrogram of transcripts of human melanoma cell lines. IL-10 is 1 of the 19 genes whose normalized expression signal was >10 in two β-catenin–accumulated cell lines and <5 in the other six. (B) IL-10 mRNA expression and protein expression in human melanoma cell lines and cultured melanocytes. IL-10 expression measured by TaqMan qRT-PCR in each cell line was normalized to an internal control transcript (GAPDH) and indicated as the relative value to that in 888mel (top). IL-10 in the culture supernatants (24 h culture of 1 × 10^6 cells) was measured by ELISA (middle). β-catenin accumulation in human melanoma cell lines and cultured melanocytes was analyzed by Western blot (bottom). *Harbor stabilizing β-catenin mutations; **lacks wild-type APC protein (10). (C) Two representative positive cases for both IL-10 and β-catenin (case A and B) and one representative negative case (case C) are shown. Sections were counterstained via Gill’s hematoxylin. Scale bars, 50 μm.
binding sites between −166 and −867 bp (Fig. 2C). The introduction of mutations in the potential TBE (5′-CATTTGTA-3′ to 5′-CAGTGGC-3′ [Mut 4047 bp–IL-10 promoter–pGL4.10]) located at −438 bp upstream of the IL-10 coding sequence reduced IL-10 promoter activity (Fig. 2C). Furthermore, ChIP assays showed endogenous β-catenin/TCF binding to the IL-10 promoter (Fig. 2D).

**FIGURE 2.** β-catenin transactivates and binds the IL-10 promoter in human melanoma cells. (A) β-catenin knockdown resulted in decreased IL-10 production from β-catenin-accumulated melanoma (624mel, 2 × 10⁹; 888mel, 1 × 10⁹; 24 h culture). The indicated melanoma cells were infected with the lentivirus encoding shRNA for either β-catenin mRNA or firefly luciferase mRNA (siGL3B; as control) or transfected with either oligonucleotide siRNA against β-catenin or scrambled sequence oligonucleotide siRNA (scramble; as control) using Lipofectamine 2000. Protein lysates were collected and immunoblotted with anti-β-catenin Ab to confirm β-catenin knockdown. (B) Overexpression of β-cateninS45Y in non-IL-10–producing melanoma cells resulted in IL-10 production. (C) A series of 5′ human IL-10 promoter deletion constructs were cotransfected with either pME-18S-β-cateninS37F or mock vector (top). Transcriptional activities of the wild-type construct and the mutant construct harboring a site-specific alteration of TBE at −438 (position 7) (bottom). (D) ChIP assays were performed in 888mel. The immunoprecipitation with the 1313–3E), indicating induction of Tregs, possibly through IL-10 production from melanoma cells, the activation of the Wnt/β-catenin signaling pathway in melanoma cells is involved in DC impairment

IL-10 was previously reported to render immature DCs to differentiate into DCregs, which promote T cell anergy and tolerance in humans and mice (20–22). IL-10–containing culture supernatants of cancer cell lines were shown to have activities suppressing the maturation of Mo-DCs (5), as well as inducing high IL-10–producing DCregs capable of inhibiting proliferation and function of T cells (23). We then evaluated whether activated Wnt/β-catenin signaling in melanoma was involved in the impairment of DCs. The addition of the culture supernatants of 624mel with mutated β-catenin (S45Y) to human Mo-DCs culture at 20% vol in the presence of IL-4 and GM-CSF for 5 d significantly reduced the production of inflammatory cytokines such as IL-12 by Mo-DCs stimulated with LPS, whereas it increased the production of IL-10, which was similar to DCreg induction with IL-10 as previously reported (Fig. 3A) (20–22). Partial recovery with scramble siRNA plus anti–IL-10 Ab may suggest possible involvement of factors other than IL-10 in the β-catenin–induced DC suppression for IL-12 production. Additionally, these suppressive activities of the melanoma supernatants were reduced by pretreatment of the melanoma cells with β-catenin RNAi (Fig. 3A). Furthermore, addition of 624mel culture supernatant not only decreased expression levels of CD1a and a co-stimulatory molecule CD86, but also increased the expression of inhibitory molecules such as ILT-3 and ILT-4, which were previously reported to be upregulated on DCregs (24) (Fig. 3B). No changes were observed regarding CD40, CD80, CD83, HLA-DR, PD-L1, and PD-L2 expression (data not shown). Concerning the function of DCs to activate T cells as APCs, the stimulatory activity of DCs on allogeneic T cells measured by production of IFN-γ from the T cells was also decreased by addition of 624mel culture supernatant (Fig. 3C). These phenotypic changes were restored by the β-catenin RNAi treatment on 624mel (Fig. 3B, 3C). Additionally, these 624mel culture supernatant-treated DCs were found to induce Foxp3+ CD4+ T cells capable of suppressing T cell proliferation (Fig. 3D, 3E), indicating induction of Tregs, possibly through IL-10 produced by the DCs or DC/T cell contact via ILT-3 or ILT-4, which had been reported to be involved in Treg induction (25, 26). Furthermore, the DCs conditioned with β-catenin RNAi–pretreated melanoma supernatant show reduced Treg induction ability (Fig. 3D). These results indicate that β-catenin/TCF directly promotes IL-10 transcription through binding to the TBE located −438 bp upstream of the coding sequence in the IL-10 promoter.

**Activation of the Wnt/β-catenin signaling pathway in human melanoma cells is involved in DC impairment**

### Table I. Positive correlation between IL-10 expression and β-catenin accumulation in human melanoma cell lines and tissues

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>β-catenin Accumulation</th>
<th>IL-10 Expression</th>
<th>p (Fisher Exact Test)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>624mel</td>
<td>4</td>
<td>0</td>
<td>0.0027</td>
</tr>
<tr>
<td>888mel</td>
<td>1</td>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>

### Table II. TBEs in human IL-10 promoter

<table>
<thead>
<tr>
<th>TBE</th>
<th>Position</th>
<th>Sequence (5′–3′)</th>
<th>Orientation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>−3839</td>
<td>AACAAAG</td>
<td>Forward</td>
</tr>
<tr>
<td>2</td>
<td>−3560</td>
<td>CATTGGAT</td>
<td>Reverse</td>
</tr>
<tr>
<td>3</td>
<td>−2999</td>
<td>ATCAATG</td>
<td>Forward</td>
</tr>
<tr>
<td>4</td>
<td>−2278</td>
<td>AACAAG</td>
<td>Forward</td>
</tr>
<tr>
<td>5</td>
<td>−2161</td>
<td>ACAAAGA</td>
<td>Forward</td>
</tr>
<tr>
<td>6</td>
<td>−1313</td>
<td>ATCAATG</td>
<td>Forward</td>
</tr>
<tr>
<td>7</td>
<td>−438</td>
<td>CATTGTA</td>
<td>Reverse</td>
</tr>
</tbody>
</table>

These results indicate that β-catenin/TCF directly promotes IL-10 transcription through binding to the TBE located −438 bp upstream of the coding sequence in the IL-10 promoter.

*-error bars are ±SD, *p < 0.05.
IL-10 also acts on murine cells (27). In this model, we implanted
We therefore adopted nude mouse xenograft models because human

cocultured with allogeneic T cells. On day 3, IFN-
were evaluated. The values are mean fluorescence intensities. Results are
DC IL-12 and IL-10 production (28–30). Interestingly, this IFN-

promoter at

FIGURE 3. Impairment of monocyte-derived DCs and induction of
possible DCregs by β-catenin-accumulated melanoma cells. Human DCs
were differentiated from CD14+ monocytes for 5 d with IL-10 or with
culture supernatants (CS) from parental 624mel (plain) or 624mel that had
be treated with β-catenin siRNA or scramble siRNA. All DC cultures
were differentiated from CD14+ monocytes for 5 d with IL-10 or with


and T cell proliferations were determined using BrdU incorporation
(Fig. 4F, 4G). Moreover, PKF115-584 treatment restored the DC functions
to activate the T cells measured by resulting IFN-

Melanoma cells with the activated Wnt/β-catenin signaling pathways inhibit effector function of melanoma-specific T cells

In vivo impairment of DCs by human melanoma cells with the activated Wnt/β-catenin signaling was then evaluated. We have attempted to make murine syngeneic melanoma models, but we failed to do so because of the lack of IL-10 production by murine melanoma cell lines, B16 and K1735, transfected with β-cateninS37F, possibly because the TBE seen in the human IL-10 promoter at −438 bp did not exist in the murine IL-10 promoter. We therefore adopted nude mouse xenograft models because human IL-10 also acts on murine cells (27). In this model, we implanted

the high IL-10–producing human melanoma cell line 397mel-β-cateninS37F, which was generated by stable transfection of β-cateninS37F using recombinant lentiviral vector (Fig. 2B). The 397mel-β-cateninS37F grew slightly less than mock transfectants, 397mel-mock, in vitro and in vivo (Fig. 4A, 4B). Human IL-10 was detected in sera from the nude mice s.c. implanted with

397mel-β-cateninS37F (Fig. 4C). T cell stimulatory activities of splenic DCs and tumor-infiltrating DCs obtained from the nude mice implanted with either 397mel-β-cateninS37F or 397mel-mock were evaluated. Both splenic DCs and tumor-infiltrating DCs from nude mice implanted with 397mel-β-cateninS37F had less stimulatory activity on T cells measured by resulting IFN-γ production when stimulated with anti-CD3 Ab than did those from mice with 397mel-mock (Fig. 4D, 4E).

Next, we evaluated whether these in vivo DC impairments by

β-catenin-activated melanoma could be reversed using a β-catenin inhibitor, PKF115-584. This inhibitor reduces Wnt reporter activity through disruption of the TCF/β-catenin complex, suppresses β-catenin target gene expressions such as c-Myc and cyclin D1, and inhibits cell growth of various β-catenin–activated cancer cells in vitro and in vivo (28–30), although it may also affect other β-catenin functions via blocking the interactions with other molecules such as E-cadherin and APCs (8). Because none of the melanoma cell lines harboring β-catenin mutations (624mel, 888mel, and 928mel) could grow in nude mice when inoculated s.c., we adopted an SCID mice model in this experiment. Based on the previous report (30), 0.08 mg/kg PKF115-584 was administered to the mice implanted with 928mel, which inhibited the IL-10 production from 928mel detected in the mice sera, indicating inhibition of β-catenin signaling in the implanted melanoma cells without affecting the cell proliferations (Fig. 4F, 4G). Moreover, PKF115-584 treatment restored the DC functions to activate the T cells measured by resulting IFN-γ production when stimulated with anti-CD3 Ab (Fig. 4H).

Taken together with the in vitro human DC study, these results indicate that the Wnt/β-catenin signaling pathway in human melanoma cells has immunosuppressive activities on DCs, leading to suppression of the induction phase of antitumor T cell responses, which could be reversed by treatments targeting β-catenin.

Melanoma cells with the activated Wnt/β-catenin signaling pathways inhibit effector function of melanoma-specific T cells

Immunosuppressive effects on effector T cells by human melanoma cells with activated Wnt/β-catenin signaling were then evaluated. When cocultured with an autologous melanoma-specific CTL, TIL397 (31), 397mel-β-cateninS37F significantly inhibited IFN-γ release from the CTLs compared with 397mel-mock (Fig. 5A). Interestingly, this IFN-γ release was not restored by addition of neutralizing anti–IL-10 Ab, indicating that Wnt/β-catenin signaling is able to suppress effector T cell function through unknown mechanisms other than IL-10. Additionally, 397mel-β-cateninS37F was more resistant to cytolysis by HLA-A1–restricted melanoma-specific allogeneic TIL1362 (Fig. 5B).

Similar resistance of 397mel-β-cateninS37F to TIL397 was demonstrated in vivo using a Winn assay by s.c. coinjection of 397mel-mock or 397mel-β-cateninS37F with TIL397 into immunodeficient NOG mice (32). The mice implanted with 397mel-β-cateninS37F along with TIL397 showed a significantly earlier tumor development and less tumor-free survival than did those implanted with 397mel-mock with TIL397 (Fig. 5C), whereas their tumor development was almost the same when inoculated without TIL397, indicating that activated Wnt/β-catenin signaling pathways in human melanoma also inhibit effector function of melanoma-specific CTLs and render melanoma cells immunoresistant to
CTL lysis. Collectively, the results in this study demonstrated that constitutively activated the Wnt/β-catenin signaling pathway in human melanoma might be involved in immunosuppression and tumor immunoresistance in both induction and effector phases of antitumor T cell responses partly through IL-10 production, and they suggest that Wnt/β-catenin in cancer cells may be a potential target for restoration of immunocompetence of patients with melanoma that exhibit increased Wnt/β-catenin signaling activity.

Discussion
One of the major reasons for the relatively weak antitumor effects of present active immunization protocols is thought to be immunosuppression, particularly in the tumor microenvironment (33). In tumor tissues, the abilities of T cells to produce IFN-γ and cytotoxic molecules such as perforin and granzymes are decreased, DC functions to activate T cells are impaired, and the various immunosuppressive cells such as DCregs and Tregs are induced (34, 35). However, the molecular mechanisms leading to the induction of these immunosuppressive molecules and cells by cancer cells have not yet been well understood. In this study, we demonstrated that constitutively activated Wnt/β-catenin signaling in human melanoma might cause the immunosuppression in the tumor microenvironment.

β-catenin accumulation has been shown in ~50% of human melanoma (Table I). Although mutations of β-catenin or the associated molecules such as APCs can account for the elevated β-catenin in melanoma cell lines (10), those mutations are not frequently observed in uncultured melanoma (11). Involvement of Wnts to stimulate the canonical Wnt/β-catenin signaling pathway has not been reported. The mechanism of frequent β-catenin ac-

FIGURE 4. In vivo DC impairment for T cell activation in the mice bearing β-catenin–activated melanoma and its recovery by a β-catenin inhibitor. (A and B) Slight decrease of in vitro (WST-1 assay) (A) and in vivo (B) cell proliferation of 397mel-β-cateninS37F. (C) Human IL-10 was detected in sera from 397mel-β-cateninS37F–implanted nude mice. (D and E) Splenic DCs (D) or tumor-infiltrating DCs (E) were obtained from untreated (no tumor), 397mel-β-cateninS37F–implanted, or 397mel-mock–implanted nude mice, irradiated, and cocultured with T cells from BALB/c mice in the presence of anti-CD3 Ab for 5 d. The results of splenic DCs (D) or tumor-infiltrating DCs (E) are from two representative experiments. IFN-γ production was determined to measure T cell activation. T cells incubated without DCs serve as negative control. (F) The production of human IL-10 was determined to be inhibited by PKF115-584 treatment. Human IL-10 in SCID mice sera was measured before treatments (Tx) and after 12-time treatments (12 Txs). (G) The proliferation of 928mel in SCID mice was not affected by PKF115-584 treatment. (H) DCs were obtained from no tumor, DMSO–, or PKF115-584 (0.08 mg/kg)–treated SCID mice, irradiated, and cocultured with T cells from BALB/c mice in the presence of anti-CD3 Ab for 5 d. IFN-γ production was determined to measure T cell activation. Error bars are ±SD. *p < 0.05.

FIGURE 5. Impairment of melanoma-specific effector CTLs by human melanoma cells transfected with β-cateninS37F. (A) 397mel-β-cateninS37F inhibited IFN-γ production from TIL397. The anti–IL-10–neutralizing Ab did not restore the IFN-γ production. (B) 397mel-β-cateninS37F was more resistant to cytolysis by allogeneic melanoma-specific CTL, TIL1362, which shared HLA-A01/25 with TIL397, in a [51Cr]-release assay. Untreated parental 397mel was included to confirm that lentiviral transfection had no effect on the lysis susceptibility. Results are representative of three experiments. (C) 397mel-β-cateninS37F was more resistant to the CTLs than was 397mel-mock in vivo in the Winn assay (tumor-free curve generated with Kaplan–Meier analysis [p value, log-rank test; top], tumor growth curve [bottom]). Error bars are ±SD. *p < 0.05.
cumulation in human melanoma tissues remains to be investigated. The activated β-catenin signaling induces MITF, which has oncogenic ability in human melanoma (36) and is involved in melanoma tumorigenesis together with an activated N-Ras in a certain mouse model (7). These observations suggested that the β-catenin is involved in melanoma development. However, the role of Wnt/β-catenin in progression of human melanoma is not well understood. Although many target genes of the Wnt/β-catenin signaling pathway in melanoma, including ubiquitous genes such as myc or cyclin D1, cell lineage-restricted genes such as Bm2, and melanocyte-specific genes such as MITF and DCT (36–38), are associated with melanoma proliferation and melanocyte differentiation and survival, there were contradictory reports regarding relationship between Wnt/β-catenin signaling activation and patient prognoses (12). In this study, melanoma cell proliferations were not inhibited by β-catenin RNAi (data not shown), although mild but significant inhibition of cell growth was observed in melanoma cells transfected with β-cateninS37F in vitro and in vivo (Fig. 4A, 4B). Overexpression of β-catenin might inhibit melanoma cell proliferation through an increase of CDK inhibitors such as p21 and p27 by upregulation of MITF via β-catenin as previously reported (39). Therefore, inhibition of the Wnt/β-catenin signaling pathway may have only a minor effect on human melanoma cell growth.

Roles of Wnt/β-catenin signaling in the immune response of human melanoma cells have not been well evaluated. In this study, we demonstrated that IL-10 was a novel target of the Wnt/β-catenin signaling pathway in human melanoma and that the constitutively activated Wnt/β-catenin signaling might contribute to the suppression of both induction and effector phases of anti-melanoma T cell responses via impairment of DC and T cell functions partly owing to production of IL-10 and generation of the immunosuppressive microenvironment. Recently, ectopic CTLa4 expression on melanoma cells has been reported to be induced by Wnt signaling (40). Although its functional roles are not yet clear, such ectopic expression of possibly immunoregulatory molecules may alter the immune responses in the tumor microenvironment along with the immunosuppression by β-catenin–accumulated melanoma presented in this study.

IL-10 expression is regulated by various mechanisms. Transcription factors such as Sp1 and STAT3 are involved in the IL-10 regulation in macrophages and DCs (41, 42). In human melanoma, we have previously reported that STAT3 and MAPK signaling pathways are involved in IL-10 production (5), although their activation is not always correlated with IL-10 production. In this study, we identified that β-catenin directly promoted IL-10 transcription through binding to the IL-10 promoter. It has recently been reported that HDAC11, a member of the HDAC histone deacetylase family, negatively regulates IL-10 expression in DCs and macrophages (43). Interestingly, this gene is located in the specific region of chromosome 3 (3p25.1–3p25.2) in which human uveal melanoma frequently had allelic loss. It remains to be investigated whether HDAC11 may also be involved in the regulation of IL-10 expression in cutaneous melanoma, particularly with β-catenin accumulation.

IL-10 exerts its immunosuppressive activities through the suppression of APCs and T cells. Our present study and those of others showed that IL-10 in the cancer cell culture supernatants was involved in the induction of DCregs (23). Increased expression of IL-10 was detected in freshly isolated human melanoma biopsies (44), and elevated levels of serum IL-10 in melanoma patients are correlated with poor survival (45). These observations support immunosuppressive roles of IL-10 in human melanoma. However, in mouse models, the effects of IL-10 on antitumor responses are controversial. Whereas several preclinical models show that IL-10 suppresses the immune responses against cancers, some studies show that IL-10 inhibits tumor growth by stimulating host immune cells such as CTLs and NK cells (46, 47). One explanation for this contradiction might be the difference of IL-10 concentrations in these models. As suggested by García-Harnández et al. (47), whereas the mouse models using IL-10 gene-transfected tumor cells producing high amounts of IL-10 (20–2000 ng/ml/10⁶ cells/48 h) showed IL-10–mediated tumor reduction, the other models with much lower concentration of IL-10 (0.016–5 ng/ml/10⁶ cells/48 h) showed that IL-10 suppressed the immune responses and increased tumor growth. In our study, 397mel-β-cateninS37F produces ∼0.7 ng/ml IL-10 (10⁶ cells/48 h) and serum concentration is ∼30 pg/ml, similar to the latter mouse models in which IL-10 enhanced tumor growth or melanoma patients (45). Thus, IL-10 observed in melanoma patients appears to have immunosuppressive roles.

The IL-10–independent mechanism for the observed inhibition of IFN-γ release from melanoma-specific CTLs by 397mel-β-cateninS37F is of interest. Previously, IL-10 pretreatment on tumor cells has been reported to downregulate the expression of HLA class I and TAP, which has an important role in Ag presentation associated with HLA, and protects the tumor cells from CTL attacks (48, 49). Moreover, IL-10 directly affects the function of T cells and inhibits IL-2, TNF-α, and IL-5 production (44). However, in our study, anti-IL-10–neutralizing Ab did not restore CTL function (Fig. 5A), the IL-10 receptor was not detected on 397mel by flow cytometry, and no change of HLA class I expression was observed between 397mel-β-cateninS37F and 397mel-mock (data not shown), indicating that IL-10 did not seem to be involved in the resistance of melanoma cells transfected with β-cateninS37F. Thus, unknown mechanisms regulated by β-catenin may exist for the CTL inhibition and are to be determined in future studies.

When considering the Wnt/β-catenin signaling pathway in melanoma cells as a potential therapeutic target to restore immunocompetence of patients, we have to consider effects of Wnt/β-catenin inhibition to other normal cells, including immune cells. Interestingly, recent studies have showed that β-catenin was involved in immune tolerance and suppression. Introduction of stabilized β-catenin into Tregs has been reported to result in a marked enhancement of survival of these cells (50). Involvement of β-catenin in the generation of murine DCregs has also been reported (51, 52). Therefore, targeting the Wnt/β-catenin signaling pathways both in melanoma and these immune cells such as Tregs and DCs may synergistically result in favorable consequences for restoration of immunocompetence of patients, suggesting that Wnt/β-catenin may be an attractive therapeutic target.

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