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Original Article

Integrin αvβ6 mediates the immune escape through regulation of PD-L1 and serves as a novel marker for immunotherapy of colon carcinoma

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Abstract: The immune escape of colon cancer and its role in the response to immunotherapies such as PD-1/PD-L1 checkpoint inhibitors have long been of great interest. The positive outcomes of immunotherapy are limited by the immunosuppressive nature of the tumor microenvironment. Integrin αvβ6, which can regulate the progression of colon cancer, was recently reported to be involved in the immune suppression of colon cancer. In the present study, we explored the correlation between αvβ6 and PD-L1 expression by immunohistochemistry of colon cancer tissues. Then, the regulation of PD-L1 signaling by αvβ6 in colon cancer cells was demonstrated. We constructed an in vivo model and performed immunophenotyping experiments to analyze further the regulation of the immune response by αvβ6. The role of αvβ6 in the response to anti-PD-1 therapy in colon cancer was also verified. αvβ6-positive tissues exhibited increased PD-L1 expression. Inhibition of αvβ6 not only downregulated constitutive PD-L1 expression but also decreased IFN-γ-induced PD-L1 expression. In addition, αvβ6-induced PD-L1 expression was suppressed by the ERK inhibitor PD98059, and knockdown of the β6-ERK2 binding site had the equivalent effect. αvβ6 decreased CD8+ T cell infiltration and granzyme B expression in CD8+ T cells in colon cancer patients. Furthermore, mice engrafted with αvβ6-expressing colon cancer cells exhibited an unsatisfactory response to anti-PD-1 therapy, and anti-PD-1-induced increases in CD4+ and CD8+ T cell infiltration could be inhibited by αvβ6. These results indicate that αvβ6 mediates immune escape in colon cancer by upregulating PD-L1 through the ERK/MAPK pathway. Moreover, αvβ6 could serve as a marker for the efficacy of anti-PD-1 therapy in colon cancer.

Keywords: Integrin αvβ6, PD-L1 expression, colon cancer, immune escape, cancer immunotherapy

Introduction

Colon cancer is one of the most common malignancies and is the second leading cause of cancer-related deaths worldwide [1]. Colon cancer prognosis has markedly improved due to advances in surgical technology, radiotherapy and the use of efficient chemotherapeutics. Nevertheless, 40-50% of colon cancer patients have an unsatisfactory outcome because of malignant progression [2]. There have been numerous breakthroughs in the development of immunotherapies, such as immune checkpoint inhibitors, for many types of human cancers. However, colon cancer is one of the few cancers that does not respond well to immune checkpoint-related immunotherapy [3]. Therefore, exploring the mechanisms of immune escape in colon cancer may provide a potential target for immunotherapeutic approaches for colon cancer and improve treatment efficacy.

The programmed death 1/PD ligand 1 (PD-1/PD-L1) axis is a major pathway regulating the immune system by balancing tumor immunity and inflammatory reactions, thus attenuating the immune response mediated by T lymphocytes that have migrated to the tumor microen-

vironment; this pathway is the subject of intense study in the field of cancer immunotherapy [4]. PD-L1, the first discovered ligand of PD-1, is expressed in cells of different lineages, including immune and tumor cells, and is considered the main factor responsible for promoting tumor immune escape [5]. PD-L1 inhibitors account for the largest proportion of drugs approved by the FDA in recent years and many others are currently in clinical trials. For colon cancer, although the PD-L1-positive rate is almost 50%, anti-PD-1 or anti-PD-L1 drugs show undesirable efficacy, except in MSI-positive patients, which represent less than 4% of colon cancer patients [6]. The poor treatment response, in addition to the high variation in genetic mutations among individuals, may also be related to the complex microenvironment of tumors. Recent studies have shown that the interaction between immune and cancer cells, certain cytokines and tumor-derived exosomes in the tumor microenvironment can induce the expression of PD-L1 and promote tumor immune escape [7-9].

Integrins belong to the cell surface adhesion molecule family and are composed of noncovalently associated α and β subunits [10]. In the tumor microenvironment, they regulate cell-cell and cell-matrix interactions through signaling and play numerous critical roles, including regulating the proliferation and migration of tumor cells [11, 12]. The β6 subunit is expressed exclusively in epithelial cells and it only forms a heterodimer with αv, called integrin αvβ6. β6 is absent or expressed only at low levels in healthy adult epithelia but is highly expressed during embryogenesis, tissue repair and carcinogenesis [13, 14]. We have previously reported that integrin αvβ6 in colon cancer promotes cell proliferation, mediates metastasis, and even contributes to chemoresistance. Furthermore, we demonstrated that the tumor-promoting effects of αvβ6 in colon cancer are mediated by directly binding to extracellular signal-regulated kinase (ERK2). Blocking this interaction obviously impaired cytosolic ERK/MAPK signaling and suppressed αvβ6-mediated proliferation and metastasis [15-17].

Integrins have been reported to be involved in tumor immune escape. For example, integrin αvβ8 binds to and supports the activation of TGF-β, which decreases the infiltration of cytotoxic T cells and proinflammatory tumor-associated macrophages into the tumor center [18]. Regarding αvβ6, a greater frequency of Treg cells and a lower frequency of CD8+ T cells were detected in αvβ6-positive colon cancer tissues than in αvβ6-negative tissues [19]. However, the precise mechanism underlying β6-mediated tumor immune escape in colon cancer or other cancers remains largely unknown.

Recent reports have indicated that PD-L1 expression in cancer cells can be significantly suppressed by an inhibitor of the MAPK pathway. Moreover, the antitumor efficacy of PD-L1 in colon cancer models may be improved by combination with a MAPK inhibitor [20]. Thus, considering the regulatory effect of αvβ6 on MAPK signaling, we hypothesize that αvβ6 regulates PD-L1 expression in colon cancer cells and is involved in immune escape. In the present study, we investigated the prognostic significance of αvβ6 and PD-L1 in colon cancer and explored the potential role and underlying mechanism of αvβ6 in PD-L1-mediated tumor immune escape in colon cancer.

Materials and methods

Clinical samples

Patients underwent radical tumor resection surgery at the Department of Gastrointestinal Surgery, Shandong Provincial Hospital, Jinan, Shandong, China, between June 2019 and July 2022. There were 79 men and 47 women with a median age of 64.6 years and an age range of 42-81 years. In addition, all colon cancer patients whose diagnoses were confirmed by histological examination must have received surgical resection as the initial treatment without major perioperative complications. Colon cancer was classified into right hemicolon cancer (including cecum, ascending colon, and right transverse colon) and left hemicolon cancer (including left transverse colon and descending colon) based on the anatomical location of the primary tumor. The pathologic tumor-node-metastasis (TNM) staging system was applied according to the classification of the International Union Against Cancer (2020). This study complied with the requirements of The Ethics Committee of Shandong Provincial Hospital, Shandong First Medical University, China (SWYX: No. 2019-115).

Colon cancer cell lines

The human colon cancer cell lines SW480, HT29, and WiDr and the mouse colon cancer cell line MC38 from ATCC were cultured in standard medium supplemented with 10% fetal bovine serum (FBS) and bovine serum at 37°C with 5% CO_2. Both the HT29 and WiDr cell lines were transfected with a β6-specific small interfering RNA (siRNA; GenePharma) or negative control in Opti-MEM (Invitrogen, Carlsbad, CA) using Lipofectamine 2000 (Invitrogen, USA) as previously described. Wild-type SW480 (β6-negative expression) cells were stably transfected with pcDNA1neo constructs containing wild-type full-length β6, a deletion mutant β6 (which lost the ERK2 binding site: 749RSKAKWQTGTNPLYR763) or the expression plasmid only (Mock) as reported previously [15]. MC38 cells were transfected with lentiviruses (GENEGHEM, China) carrying the constructs for the overexpression of β6.

Bioinformatics analysis

RNA-sequencing expression profiles and corresponding clinical information for advanced colon cancer patients were downloaded from the TCGA dataset (ITGB6 high: n=106; ITGB6 low: n=86). Tumor-infiltrating immune cells were identified based on CIBERSORT, a gene expression-based deconvolution algorithm that uses a set of barcoded gene expression values to characterize immune cell composition. Moreover, SIGLEC15, TIGIT, CD274, HAVCR2, PDCD1, CTLA4, LAG3 and PDCD1LG2 were selected as immune checkpoint-related genes, and the expression levels of these eight genes were determined.

All of the above analysis methods and R packages were implemented with the R Foundation for Statistical Computing software using the ggplot2 R package.

Immunohistochemistry

Tissues were obtained from formalin-fixed paraffin-embedded tissue blocks and were selected based on hematoxylin-eosin (HE) staining. Paraffin sections were dewaxed, followed by antigen retrieval with 10 μmol/L citrate buffer (pH=6.0). Deparaffinized sections were treated with methanol containing 3% hydrogen peroxide for 15 min. After washing with PBS, the sections were incubated with blocking serum for 30 min, followed by incubation with anti-β6 (HPA023626, Merck Millipore, Inc., US), anti-PD-L1 (ab205921, Abcam, US) or anti-CD8 (sc-1181, Santa Cruz Biotechnology, US) antibodies overnight at 4°C. On the second day, the tissues were incubated with the universal IgG antibody-Fab-HRP polymer (ab996, Absin, China) for 30 min. Subsequently, the sections were stained with diaminobenzidine and hematoxylin. Normal mouse IgG was substituted for the primary antibody as the negative control. Finally, the samples were observed under a light microscope (Olympus Corp, Japan).

Immunohistochemical staining for αvβ6 and PD-L1 was evaluated according to previously described methods with modifications. The intensity of positive cells was evaluated based on the color of the positive cells and was scored as 0 (no staining), 1 (weak), 2 (moderate), or 3 (strong). The density of positive cells was scored as 0 (staining % ≤5%), 1 (5%-30%), 2 (30%-70%), or 3 (>70%). The product of the intensity and extent scores was used as the final immunohistochemistry score, which ranged from 0 to 12. Patients with a final score of 0-3 were assigned to the negative expression group, and those with a final score >3 were assigned to the positive expression group.

Flow cytometry

Harvested cells were washed with phosphate-buffered saline (PBS) and blocked with goat serum at 4°C for 30 min. Then, the cells were incubated with an anti-αvβ6 monoclonal antibody (MAB2074, Merck Millipore, Inc., US) for 20 min at 4°C and washed twice with PBS. For αvβ6 analysis, the cells were then incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG for 20 min at 4°C. Then, the cell concentration was adjusted to 1×10^6 cells/ml prior to flow cytometry analysis (FACSCalibur; BD, USA).

Quantitative real-time PCR

HT-29 and WiDr cells were treated as described above. Total RNA was extracted with TRIzol (Invitrogen, USA). Reverse transcription was performed using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, USA) according to the manufacturer's protocols. cDNA obtained from the reverse transcription reaction was

analyzed by a real-time PCR thermocycler. Quantitative values were obtained by the threshold cycle value. The relative mean fold change in expression ratios was calculated by the $2^{-\Delta\Delta Ct}$ method. The primers for human integrin PD-L1 were as follows: 5'-TATGGTGGTGCCGACTACAA-3' (forward) and 5'-TGCTTGTCCAGATGACTTCG-3' (reverse). The housekeeping gene GAPDH served as an internal control.

Western blot analysis

Cells were treated with RIPA lysis buffer containing phenylmethylsulfonyl fluoride, protease inhibitors, and phosphatase inhibitors. A BCA Protein Assay Kit (Thermo Fisher Scientific, USA) was used to quantify the protein levels. After boiling for 10 min, samples with equal amounts of protein were loaded onto an SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) gel and electrophoresed. Subsequently, the separated proteins were transferred onto polyvinylidene fluoride membranes. The membranes were incubated with primary antibodies overnight at 4°C, followed by incubation with horseradish peroxidase - labeled secondary antibodies for 1 h at room temperature. Immunoreactive bands were visualized using chemiluminescence (Millipore, USA), and the optical density was analyzed with ImageJ. The values are expressed as the fold change between the target value and the GAPDH value.

In vivo experiments

6-8-week-old female C57BL/6 mice were purchased from Beijing Vital River Laboratory Animal Technologies, China. For the tumorigenesis assay, $MC38^{WT}$, $MC38^{\beta6}$, and $MC38^{Mock}$ mouse colon cancer cells (1×10^5 cells/mouse) were injected into the subcutaneous flank of C57BL/6 mice after one week of adaptive feeding. One week after injection, the tumor was well established, and its volume was monitored by measuring the tumor diameter with electronic calipers every other day. Volumes were calculated using the formula (length) × $(width)^2/2$. The mice were sacrificed 2 weeks later or when the tumor volume exceeded 1000-2000 mm^3, ulceration occurred, or the animals exhibited distress or pain. The tumor was removed from the mouse's body, its volume and weight were measured, and immunophenotyping was performed.

To determine the efficacy of ani-PD-L1, $MC38^{WT}$ and $MC38^{\beta6}$ mouse colon cancer cells were injected into the subcutaneous flank of C57BL/6 mice as described above. The tumors were allowed to grow for 14 days. Mice selected for the experiment had a tumor size of at least 0.5 cm in diameter and were randomized to different antibody treatment groups. The mice were weighed daily, and the tumor sizes were measured every other day for 30 days. The mice were euthanized when the tumor size exceeded 2000 mm^3 or when there was large ulceration at the tumor site. Depending on the group, 10 mg/kg anti-PD-L1 (BE0361, Bioxcell, USA) or isotype control antibodies were injected on days 0, 4, and 8 of the experiment. For the immunophenotyping experiments, the tumors were harvested 4 days after the last treatment. All animal studies were performed according to the protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Shandong Provincial Hospital, Shandong First Medical University, China.

Immunophenotyping experiment

Single tumor cells used for surface and intracellular staining were isolated as described previously [18]. In brief, the subcutaneous tumors were removed from the mice and minced using sterile scissors. Then, the tissues were placed in a digestion cocktail of 2 mg/mL collagenase XI, 0.5 mg/mL hyaluronidase, and 0.1 mg/mL DNase prepared in C10 media (RPMI 1640, 1% HEPES, 10% fetal calf serum, 1% penicillin/streptomycin, 1 mM sodium pyruvate, 1% non-essential amino acids, and 0.45% beta-mercaptoethanol). The tissues were incubated in a shaker at 255 rpm for 45 minutes at 37°C. After incubation, the digested tumor cells were gently vortexed for 15 seconds. The cell slurry was passed through a 100 μm mesh strainer, and the cells were resuspended in C10 media at 12 million/mL. Then, 200 mL of cell suspension was transferred to round-bottom 96-well plates per well and incubated overnight in 5% CO_2 at 37°C. Tonbo Cell Stimulation Cocktail was added to the cells, which were incubated in a tissue culture incubator in 5% CO_2 at 37°C for 4 hours. The cells were pelleted by centrifugation for 5 minutes at 200 × g at 4°C and resuspended in PBS, followed by transfer to each well of a v-bottom 96-well plate.

The cells were incubated with a Ghost antibody for 20 minutes at 4°C. Fc receptor and nonspecific binding were blocked with anti-CD16/CD30 for 10 minutes at 4°C. Surface staining was performed for 20 minutes at 4°C. For intracellular staining, the cells were incubated in Fix/Perm buffer for 20 minutes at room temperature, followed by intracellular cytokine staining with antibody cocktails for 20 minutes at 4°C. Finally, the cells were transferred to FACS buffer (PBS with 2% FBS, penicillin/streptomycin/glutamate, and EDTA 2 mM) for analysis.

The following antibodies were used for cell surface and intracellular staining: Ghost Dye™ Violet 510 (Tonbo, USA), CD45.1-AF700 (110723, Biolegend, USA), CD8-BV605 (104729, Biolegend, USA), CD4-BV650 (100546, Biolegend, USA), IFNγ-FITC (11-7311-82, eBioscience, USA), Granzyme B-PE (12-8896-42, eBioscience, USA), CD3-APC (Biolegend, USA), and IL-17A-PE-Cy7 (25-7169-41, eBioscience, USA).

Statistical analysis

A chi-square test of cross-tabulations and Fisher's exact test were applied to analyze the relationship between the expression levels of αvβ6 and PD-L1. Survival analyses were conducted by the Kaplan-Meier method and the log-rank test. Statistical comparisons were made by one-way ANOVA or t tests. The data are presented as the means ± SDs; all measurements were obtained from at least three independent experiments. $P<0.05$ was considered to indicate statistical significance. Statistical analyses were performed using GraphPad Prism.

Results

The expression of integrin αvβ6 is associated with distinct immune phenotypes and positively correlates with PD-L1 expression in colon cancer

To determine whether integrin αvβ6 is involved in immune microenvironment regulation in colon cancer, we divided the patient cohort into ITGB6-high and ITGB6-low groups and then comprehensively analyzed the status of immune cell infiltration in colon cancer by CIBERSORT analysis. The proportions of 22 types of adaptive and innate immune cells in each sample were analyzed using the TCGA database. M2 macrophages were the most enriched immune cells in the ITGB6-high group, while CD8+ T cells and Tregs were significantly decreased ($P<0.05$, **Figure 1A**). We speculated that integrin αvβ6 is involved in immune microenvironment regulation in colon cancer, so the associations of αvβ6 with the 8 main immune checkpoint-related genes were also analyzed using datasets from the TCGA database. Bioinformatics analysis revealed a significant correlation between ITGB6 and CD274 (PD-L1), HAVCR2, and PDCD1LG2 expression in colon cancer ($P<0.05$, **Figure 1B**). Moreover, the expression levels of ITGB6 and CD274 (PD-L1) were significantly correlated in multiple tumor tissues, such as colon cancer, bladder cancer, prostate adenocarcinoma and thyroid cancer (**Figure 1C**).

Immunohistochemistry for the expression of integrin αvβ6 and PD-L1 in colon cancer

Integrin αvβ6 and PD-L1 expression in colon cancer was determined by immunohistochemistry. Both αvβ6 and PD-L1 staining were observed predominantly in the membrane of tumor cells. For 126 primary colon cancer samples, positive αvβ6 expression was detected in 53 patients (42%), and positive PD-L1 expression was detected in 57 patients (45.2%) (**Figure 1D**; **Table 1**). The relationships between αvβ6 and PD-L1 expression and clinicopathological factors of the 126 patients with colon cancer were also investigated. The number of late T stage (T3, T4) and late N stage (N1, N2) tumors was much greater than that of early T stage and N stage tumors among αvβ6-positive specimens. Accordingly, αvβ6-positive patients had a greater percentage of TNM stage III-IV tumors. PD-L1 expression was also strongly associated with the T stage, N stage and TNM stage of tumors. Moreover, αvβ6-positive/PD-L1-positive specimens exhibited a greater proportion of poorly differentiated tumors than tumors with other expression patterns (**Table 1**).

PD-L1 expressed in tumor cells has been suggested to mediate immune evasion, as PD-L1 suppresses the infiltration of CD8+ T cells into the tumor microenvironment. Consistently, in the immunohistochemistry study, PD-L1-positive colon cancer patients were found to have a lower percentage of high CD8+ T infiltrating

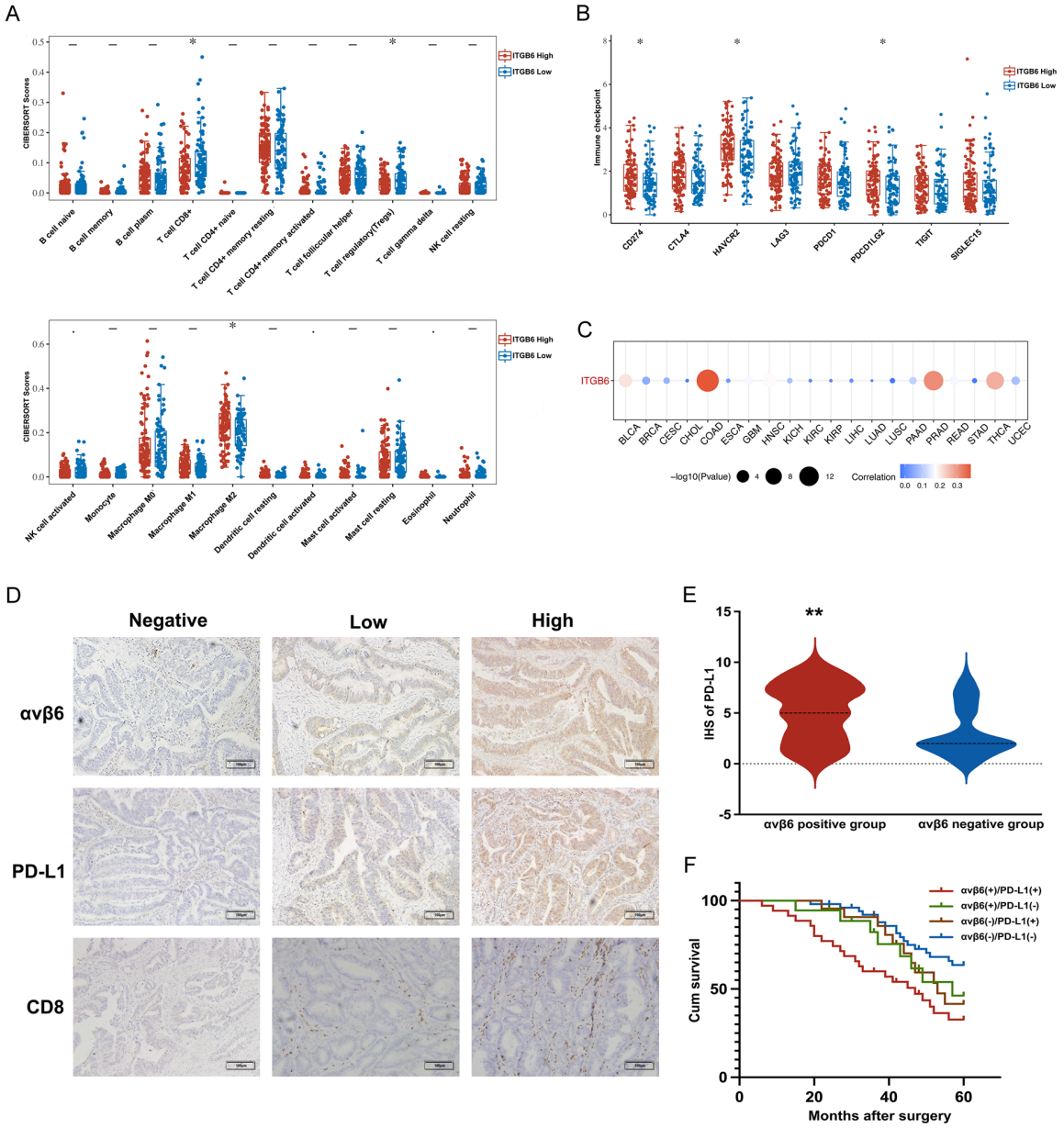

Figure 1. Analysis of the association between αvβ6 expression with immune cell infiltration and PD-L1 expression. A. The difference of immune infiltration between αvβ6-low and αvβ6-high expression group by using CIBERSORT analysis. *P<0.05, **P<0.01 versus αvβ6-low patients. B. Correlation of αvβ6 and immune checkpoint relevant genes in TCGA colon cancer. High expression of αvβ6 was correlated positively with PD-L1 (CD274). *P<0.05, **P<0.01 versus αvβ6-low patients. C. pan-cancer analysis of the correlation between αvβ6 and PD-L1 expression in different cancer subtypes. D. Immunohistochemical expression of integrin αvβ6, PD-L1 and CD8+ T cells in colon cancer tissues. Bar =100 μm. E. Association between αvβ6 and PD-L1 expression in colon cancer patients. The box-and-whisker graph demonstrated that the IHS of PD-L1 in the αvβ6-positive group was significantly higher than that in the αvβ6-negative group. **P<0.01 versus the αvβ6-negative group. F. Association between αvβ6 and PD-L1 expression and the overall survival of colon cancer patients.

lymphocytes (TILs) than PD-L1-negative patients (P=0.007). Additionally, we determined that there was a significant correlation between integrin αvβ6 expression and CD8+ T lymphocyte density in colon cancer patients (P=0.019). Among αvβ6-positive cases, a low CD8+ TIL density was detected in 62.3% (**Table 1**).

Table 1. Relation between integrin αvβ6, PD-L1 expression and clinicopathologic variables in colon cancer cases

Clinicopathological factors	n	αvβ6 expression		*P* Value	PD-L1 expression		*P* Value
		Positive (n=53)	Negative (n=73)		Positive (n=57)	Negative (n=69)	
Gender				0.159			0.311
Male	79	37	42		33	46	
Female	47	16	31		24	23	
Age (years)				0.840			0.229
≤60	70	30	40		35	35	
>60	56	23	33		22	34	
Tumor anatomical location				0.429			0.912
Right hemicolon cancer	67	26	41		30	37	
Left hemicolon cancer	59	27	32		27	32	
T stage				0.018			0.005
T1	7	3	4		2	5	
T2	20	5	15		10	10	
T3	54	18	36		16	38	
T4	45	27	18		29	16	
N stage				0.007			0.043
N0	53	15	38		17	36	
N1	45	20	25		25	20	
N2	28	18	10		15	13	
M stage				0.245			0.436
M0	99	39	60		43	56	
M1	27	14	13		14	13	
TNM stage				0.006			0.010
I-II	51	14	37		16	35	
III-IV	75	39	36		41	34	
Differentiation				0.004			0.005
Well	40	10	30		15	25	
Moderate	41	16	25		13	28	
Poor/undifferentiated	45	27	18		29	16	
CD8+ TILs				0.019			0.007
Low	63	33	30		36	27	
High	63	20	43		21	42	
Survival (60-month follow-up)				0.042			0.015
Death	58	30	28		33	25	
Censored	68	23	45		24	44	

Correlation between integrin αvβ6 and PD-L1 expression in colon cancer

To explore the relationship between αvβ6 expression and PD-L1 expression, 126 primary colon cancer samples were divided into two groups according to αvβ6 expression, and the immunohistochemistry scores (IHSs) of PD-L1 were compared between the two groups by the Mann-Whitney test. A box and whisker graph demonstrated that the αvβ6-positive group exhibited greater PD-L1 expression than did the αvβ6-negative group (P<0.01, **Figure 1E**).

Furthermore, the 126 patients included in this study were divided into four groups according to their expression of αvβ6 and PD-L1Kaplan-Meier analysis indicated that patients with pos-

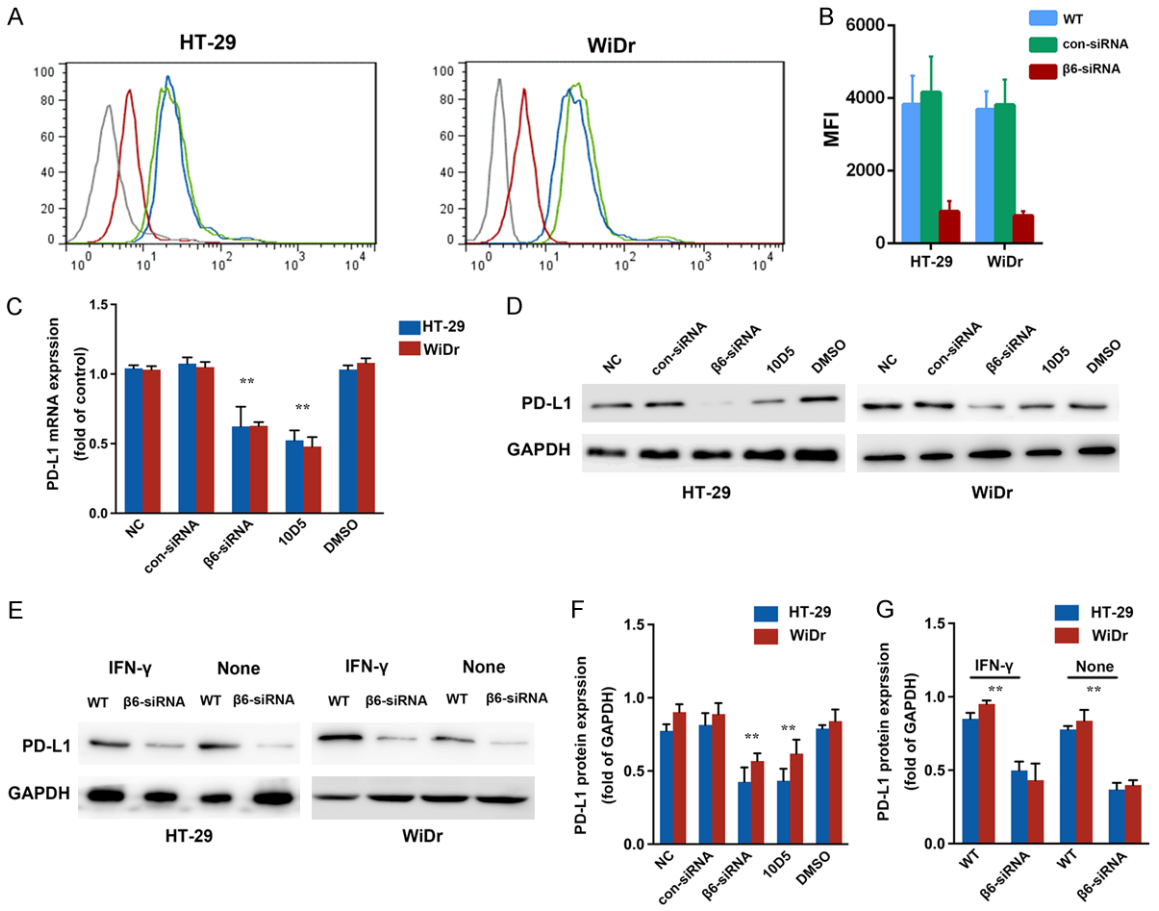

Figure 2. αvβ6 upregulated PD-L1 expression in colon cancer cells. A. Flow cytometry analysis of the surface expression of αvβ6 on HT-29 and WiDr colon cancer cells. Both of these cell lines exhibited high levels of αvβ6 expression, which could be obviously suppressed by β6-siRNA. The gray histograms represent isotype IgG controls. B. MFI quantification of αvβ6 is shown. MFI, mean fluorescence intensity. C and D. HT-29 and WiDr cells were treated with β6-siRNA and β6 antibody 10D5, and the expression of PD-L1 was analyzed by real-time PCR and western blotting. E. HT-29 and WiDr cells were pretreated with β6-siRNA, followed by stimulation with IFN-γ. Then, the expression of PD-L1 was determined by western blotting. F and G. Quantification of PD-L1 expression detected by western blot is shown. Values are expressed as fold change between the target value and the GAPDH value. **P<0.01 versus control.

itive αvβ6 and PD-L1 expression had a significantly shorter overall survival rate than patients in the other groups (P=0.026; log-rank test: χ^2=10.041). Survival over time in patients grouped based on αvβ6 and PD-L1 expression is illustrated in **Figure 1F**.

Integrin αvβ6 induces the upregulation of PD-L1 expression and regulates IFNγ-induced PD-L1 expression in colon cancer cells

We first examined the expression of αvβ6 in the HT-29 and WiDr colon cancer cell lines before investigating the crosstalk between αvβ6 and PD-L1. With respect to cell surface presentation, flow cytometry showed high membrane levels of αvβ6 in HT-29 and WiDr cells, and αvβ6 expression was obviously suppressed by β6-siRNA (**Figure 2A** and **2B**). Our abovementioned results demonstrated a correlation between the expression of αvβ6 and PD-L1 in colon cancer tissues. Therefore, we investigated the effect of αvβ6 on PD-L1 expression on colon cancer cells. Real-time PCR analysis demonstrated that inhibiting αvβ6 with siRNA significantly decreased the mRNA levels of PD-L1 in HT-29 and WiDr cells, which could be similarly downregulated by the β6-specific antibody 10D5 (**Figure 2C**). Moreover, inhibition of αvβ6 by 10D5 or siRNA significantly decreased PD-L1 expression at the protein level, which

was confirmed by western blotting (**Figure 2D** and **2F**).

PD-L1 is expressed constitutively, or its expression is induced by interferon (IFN), especially IFN-γ, in a cell line-dependent fashion [21]. To further explore whether αvβ6 was needed for IFN-γ-mediated PD-L1 expression in colon cancer cells, we examined the effects of αvβ6 depletion on PD-L1 expression under IFN-γ treatment. IFN-γ-induced PD-L1 expression was dramatically inhibited in HT-29 and WiDr cells with αvβ6 depletion by siRNA (**Figure 2E** and **2G**). We concluded that αvβ6 positively regulates constitutive and IFN-γ-induced PD-L1 expression in colon cancer cells.

Integrin αvβ6 induces PD-L1 upregulation through activation of the ERK/MAPK pathway in colon cancer cells

We then investigated the mechanism by which αvβ6 induces PD-L1 upregulation. Our previous studies confirmed that αvβ6 promoted the proliferation and metastasis of colon cancer through signaling involving the activation of the ERK pathway [17, 22]. In the present study, western blot analysis demonstrated that the expression of p-ERK was positively associated with the expression of αvβ6 in both HT-29 and WiDr cells, which was similar to our previous results. Inhibition of αvβ6 by β6-siRNA significantly reduced ERK phosphorylation, which could also be suppressed by PD98059 (a specific ERK inhibitor) (**Figure 3A** and **3C**). Moreover, inhibition of ERK phosphorylation by β6-siRNA or PD98059 significantly reduced PD-L1 expression in HT-29 and WiDr cells (**Figure 3A** and **3B**). These data indicated that the ERK pathway was likely involved in αvβ6-induced PD-L1 upregulation.

We previously demonstrated a direct link between ERK2 and αvβ6 in colon cancer cells that contributed to the activation of ERK. In this study, stably transfected SW480 cells with a mutant β6 (which lacks the ERK2 binding site) were used to investigate the mechanism of αvβ6-induced PD-L1 expression. First, western blot analysis showed that PD-L1 could be upregulated in SW480 colon cancer cells transfected with β6 (β6-OE) (**Figure 3D** and **3F**). Second, ERK phosphorylation decreased in mutant β6-transfected cells compared with that in β6-OE cells transfected with full-length β6 (**Figure 3D** and **3E**). Finally, the loss of the ERK2 binding site in αvβ6 largely reversed αvβ6-induced PD-L1 upregulation in SW480 cells (**Figure 3D** and **3F**). Therefore, it could be concluded that αvβ6 regulates PD-L1 expression through the activation of ERK, which is mediated by the direct binding of β6-ERK2. Additionally, the levels of both phosphorylated ERK and PD-L1, which were inhibited by β6-siRNA in HT-29 and WiDr cells, were obviously increased upon treatment with an ERK-activating agonist (PMA) (**Figure 3G-I**).

Furthermore, whether αvβ6-mediated ERK activation is involved in IFN-γ-induced PD-L1 expression in colon cancer cells was also investigated. Western blot experiments showed that the addition of PD98059 effectively reversed the IFN-γ-induced increase in PD-L1. In addition, PD-L1 expression was obviously suppressed by αvβ6-siRNA, which was associated with decreased phosphorylation of ERK (**Figure 3J-L**). It has been well reported that PD-L1 expression is triggered by IFN-γ mainly through the STAT1 pathway. However, neither β6-siRNA nor PD98059 significantly affected the activation of STAT1 (**Figure 3J** and **3M**). Taken together, these results indicated that αvβ6 regulates the ERK/MAPK pathway through its direct binding to ERK2, which contributes to constitutive and IFN-γ-induced PD-L1 expression in colon cancer cells.

Integrin αvβ6 promotes tumor growth in colon cancer and regulates PD-L1 expression and the immune response in vivo

To determine whether αvβ6 regulates PD-L1 expression in colon cancer in vivo and contributes to tumor immune evasion, the MC38 mouse colon cancer cell line syngeneic with C57BL/6 mice was used. MC38WT cells characterized by low constitutive αvβ6 expression were transfected with a β6-expressing lentivirus to generate MC38β6 cells stably expressing αvβ6. MC38Mock cells that received control lentivirus were used as controls. With respect to cell surface presentation, flow cytometry showed that high membrane levels of αvβ6 were detected in MC38 β6 cells, whereas hardly any αvβ6 was detected in MC38WT and MC38Mock cells (**Figure 4A**). Compared with MC38WT and MC38Mock cells, MC38β6 cells exhibited a strong increase in

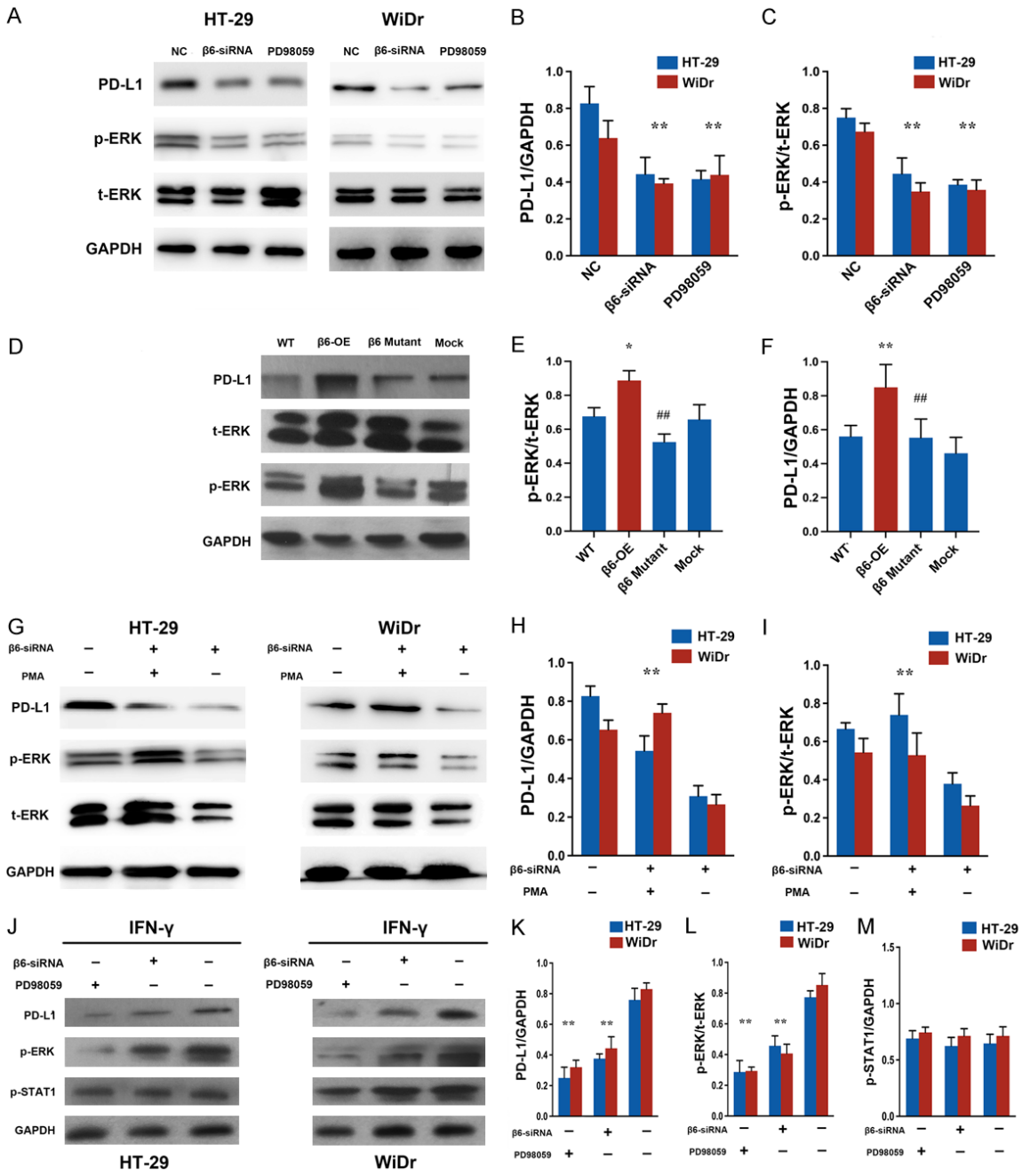

Figure 3. αvβ6 induced PD-L1 expression by activation of ERK which depends on its direct binding to ERK2. A. Expression of PD-L1, phosphorylated ERK and total ERK in HT-29 and WiDr colon cancer cells was determined by western blotting. Both phosphorylated ERK and PD-L1 expression could be inhibited by β6-siRNA or PD98059. B and C. Quantification of PD-L1 and phosphorylated ERK expression detected by western blotting. **P<0.01 versus control. D. Expression of PD-L1, phosphorylated ERK and total ERK in SW480 colon cancer cells. Knocking out the β6-ERK2 binding site decreased p-ERK and PD-L1 levels, which were increased by αvβ6. E and F. Quantification of phosphorylated ERK and PD-L1 expression detected by western blotting. *P<0.05, **P<0.01 versus control. ##P<0.01 versus β6 OE. G. The expression of PD-L1, phosphorylated ERK was examined by western blot when HT-29 and WiDr cells were treated with β6-siRNA or PMA. Inhibition of phosphorylated ERK and PD-L1 by β6-siRNA were comparably reversed when treated with PMA. H and I. Quantification of phosphorylated ERK and PD-L1 expression detected by western blotting. **P<0.01 versus PMA negative. J. HT-29 and WiDr cells were pretreated with PD98059 or β6-siRNA and the expression of PD-L1, phosphorylated ERK and phosphorylated STAT1 was examined by western blot under the condition of IFN-γ. K-M. Quantification of PD-L1, phosphorylated ERK and phosphorylated STAT1 expression is shown. **P<0.01 versus control.

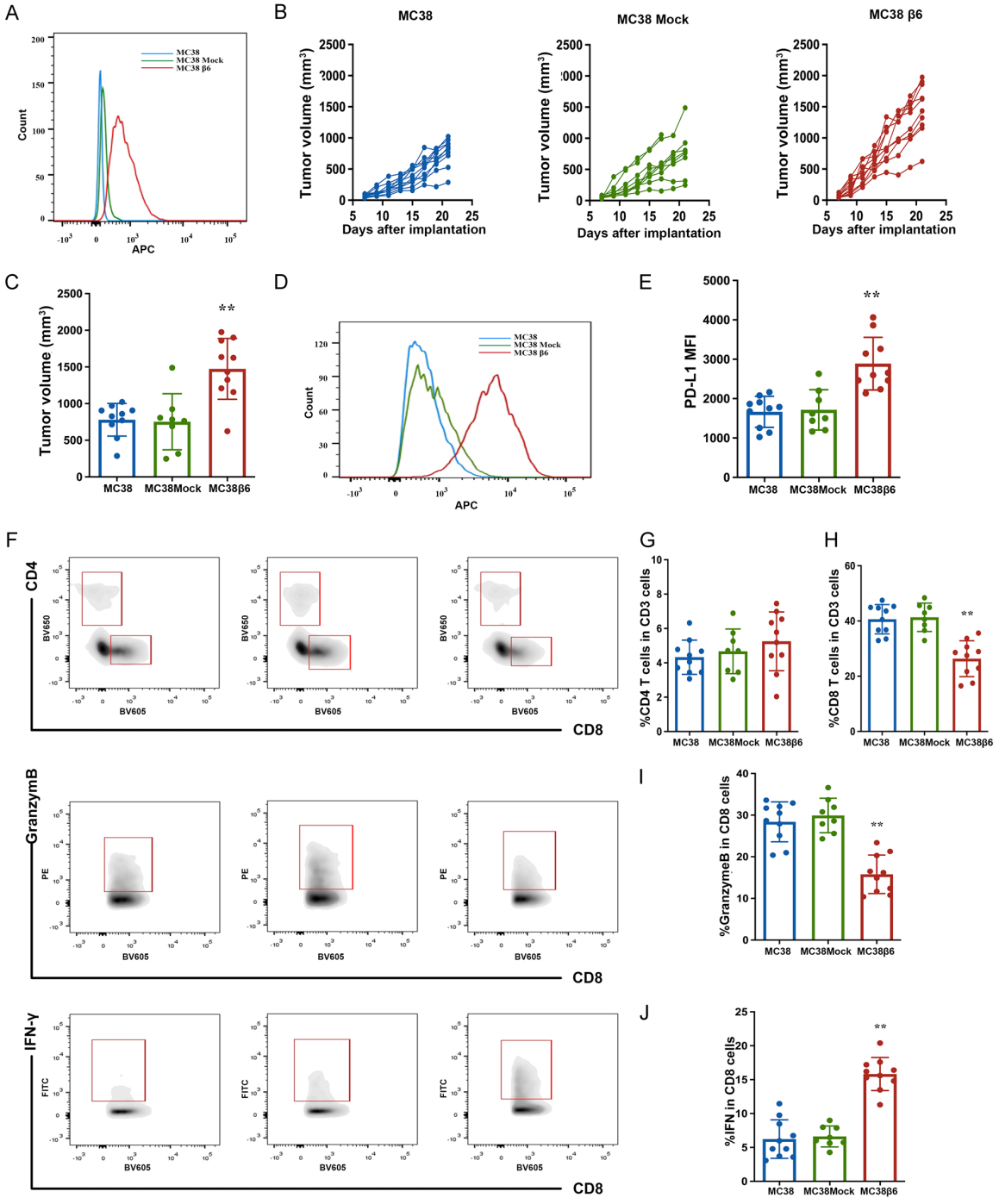

Figure 4. αvβ6 significantly promoted tumor growth, upregulated the expression of PD-L1 in colon cancer in vivo and suppressed the anti-tumor immune response. (A) Flow cytometry analysis of αvβ6 expression on MC38 mouse colon cancer cells. Wide-type MC38 cells had rare αvβ6 expression, while MC38β6 presented a high level of αvβ6. (B) Individual tumor growth curves in mice implanted with subcutaneous MC38, MC38β6 and MC38Mock cells. After growth for one week, tumor volume was well monitored for 2 weeks every other day. (C) The individual tumor volume after 3 weeks of growth is also shown. (D) Implanted tumors were harvested, and the expression of PD-L1 on tumor cells was analyzed by flow cytometry. PD-L1 expression in MC38β6 tumors was higher than that in the other groups. (E) MFI quantification of PD-L1 is shown. MFI, mean fluorescence intensity. (F) Flow cytometry was performed to gate populations of CD8+ and CD4+ T cells in tumor tissues. (G, H) Percentage of total CD8+ and CD4+ T cells in each mouse with MC38 tumors. Flow cytometry gating strategy for granzyme B (I)- and IFN-γ (J)-expressing CD8+ T cells. Percentage of granzyme B (I)- and IFN-γ (J)-expressing CD8+ T cells in each mouse. n=10 in each group. **P<0.01 versus the MC38 mouse group.

tumor growth in C57BL/6 mice (**Figure 4C**). The growth curve also indicated that mice implanted with MC38 cells transfected with β6 presented an obvious increase in tumor growth: the tumor volume of 5/10 mice was greater than 1500 mm^3 21 days after injection. Comparatively, most mice transplanted with MC38WT or MC38Mock cells showed lower levels of tumor growth (**Figure 4B**). The tumors in mice from the MC38β6 arm exhibited greater PD-L1 expression than those from the MC38WT and MC38Mock arms; hence, the upregulation of PD-L1 by αvβ6 was maintained in colon cancer in vivo (**Figure 4D** and **4E**).

Thus, since PD-L1 is a master regulator of the immune response, we speculated that the promotion of colon cancer tumor growth by αvβ6 was attributable to PD-L1-dependent immune dysregulation. Therefore, we also explored the effect of αvβ6 on the tumor immune response in this model system. The implantation of MC38β6 tumor cells resulted in an inhibitory immune response, as indicated by a decrease in CD8+ T cell infiltration. However, there was no significant difference in CD4+ T cell accumulation among the three groups (**Figure 4F-H**). In addition, αvβ6 significantly reduced the granzyme B expression rate among CD8+ T cells (**Figure 4F** and **4I**). However, IFN-γ expression in CD8+ T cells was comparably increased in the MC38β6 group (**Figure 4F** and **4J**). Therefore, it could be concluded that αvβ6, which upregulates PD-L1 expression in colon cancer cells, can suppress the tumor immune response not only by reducing the infiltration of CD8+ T cells in the tumor microenvironment but also by inhibiting its antitumor activity.

Integrin αvβ6 decreases the efficacy of anti-PD-1 therapy in colon cancer in vivo

Finally, we investigated the effect of αvβ6 on the response to PD-1 blockade immunotherapy in established syngeneic tumor models of colon cancer. We injected MC38 or MC38β6 cells subcutaneously into C57BL/6 mice and allowed the tumors to grow for 2 weeks before beginning antibody therapy. Mice were then injected with PD-1 or isotype-matched control antibody 14, 18, or 22 days after tumor implantation. The tumor diameter was measured every other day, and the mice were euthanized when the tumors reached a size greater than 2000 mm^3 (**Figure 5A** shows a schematic of the experimental design). As shown in the growth curve in **Figure 5C**, all mice that received PD-1 antibody treatment exhibited a significant, temporary reduction and delay in tumor growth. However, between the two anti-PD-1 treatment groups, there was a less significant reduction and delay for the MC38β6 mice. With anti-PD-1 treatment, 6/10 mice in the MC38 group had small tumors (<1500 mm^3), and 3/10 of the mice died after 44 days. Comparatively, for the MC38β6 group only 3/10 of mice survived after 44 days, and the other mice showed rebound growth on approximately the 28th day. The Kaplan-Meier survival curve highlighted the statistically significant advantages of anti-PD-1 therapy and the negative effects of αvβ6 therapy in the MC38 tumor model (**Figure 5B**).

To gain mechanistic insight into this inhibitory effect of αvβ6 on anti-PD-1 therapy efficacy, we analyzed tumor specimens by immunophenotyping. It is well known that immune checkpoint blockade therapy, such as anti-PD-1 therapy, suppresses tumors by increasing the number of tumor-infiltrating immune cells in the tumor microenvironment. We found that the samples from the mice that received the anti-PD-1 treatment exhibited greater infiltration of both CD4+ and CD8+ T cells, which could be inhibited by the upregulation of αvβ6 expression (**Figure 5D-F**). Moreover, granzyme B in CD8+ T cells, which was upregulated by anti-PD-1, was significantly decreased in the MC38β6 arm (**Figure 5G**). However, αvβ6 was found to increase the IFN-γ level in CD8+ T cells treated with an anti-PD-1 antibody, which was consistent with the results described above (**Figure 5H**). In conclusion, we confirmed that the formation of an immunosuppressive microenvironment by αvβ6 expression inhibited the effect of anti-PD-1 therapy on colon cancer in vivo.

Discussion

Integrin αvβ6 is an important adhesion receptor on colon cancer cells and is involved in several aggressive cell behaviors and processes, including cell adhesion and spreading on fibronectin, tumor growth, apoptosis, matrix metalloproteinase secretion and chemoresistance [14]. In our previous study, we demonstrated that the expression of αvβ6 in colon cancer tissues was closely associated with the number of tumor-infiltrating T cells [23]. In addition, it has also been reported that αvβ6 promotes tumor

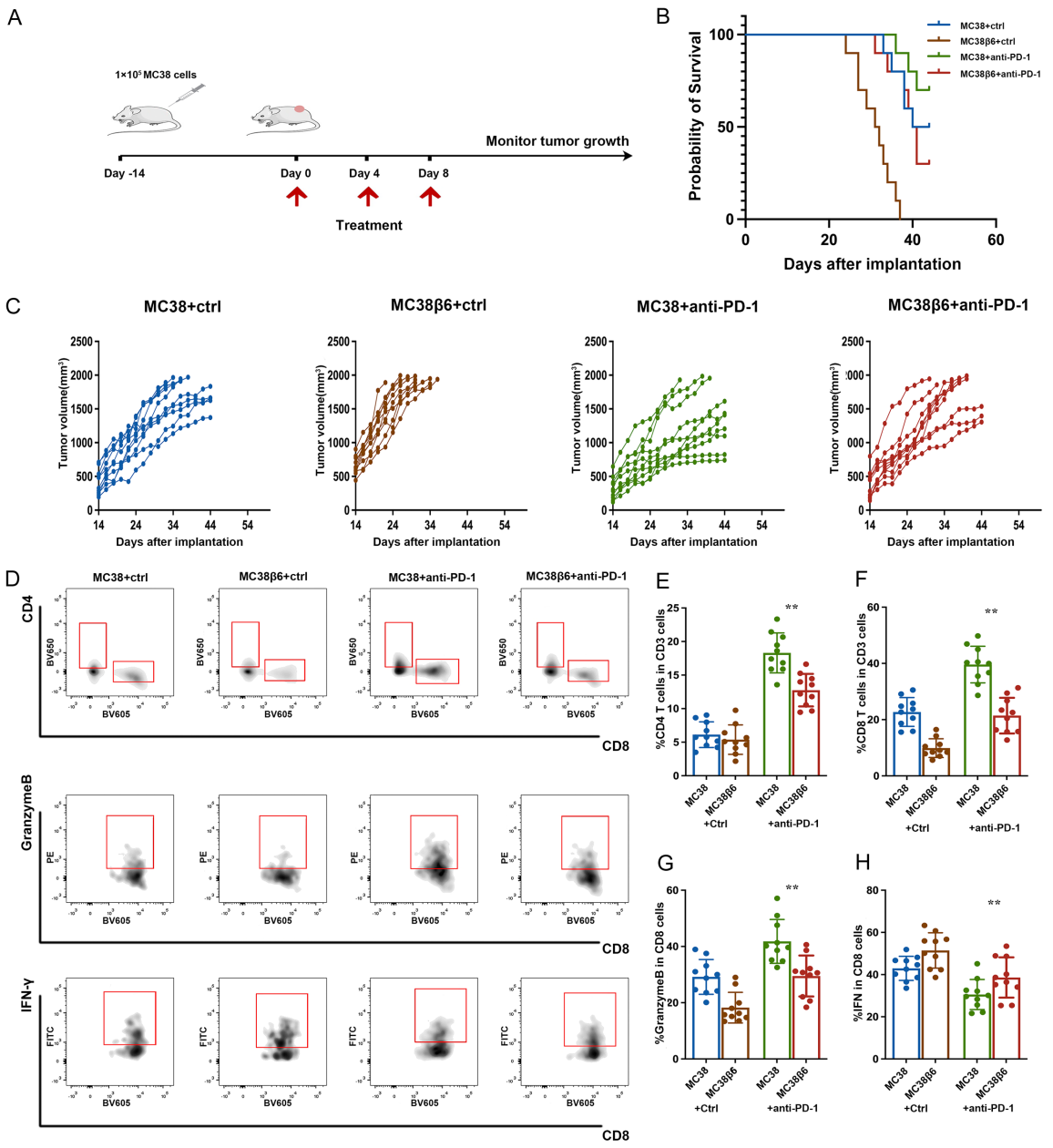

Figure 5. αvβ6 reduced the anti-tumor efficacy of anti-PD-1 therapy in colon cancer *in vivo*. (A) Schematic of the experimental design. Red arrows indicate anti-PD-1 or control antibody administration. Tumors were allowed to grow over 14 days. Antibodies for each group were injected on days 0, 4, and 8 of the experiment. Mice were weighed daily, and tumor size was measured every other day. Mice were euthanized when the tumor size exceeded 2000 mm^3 or large ulceration at the tumor site. (B) Kaplan-Meier survival curve of MC38 and MC38β6 mice treated with anti-PD-1 or control antibody. (C) Kinetics of individual tumor growth 30 days after anti-PD-1 or control antibody administration. (D) Populations of CD8+ and CD4+ T cells in tumor tissues were detected by flow cytometry. (E and F) Percentage of total CD8+ and CD4+ T cells in tumor tissues of MC38 mice. Flow cytometry gating strategy for granzyme B (G)- and IFN-γ (H)-expressing CD8+ T cells. (G and H) Percentage of granzyme B- and IFN-γ-expressing CD8+ T cells in each mouse. n=10 in each group. **P<0.01 versus the MC38 mouse group.

immune evasion in colon cancer by increasing the infiltration of Treg cells and suppressing the infiltration of tumor-infiltrating CD8+ T cells [19]. However, the mechanism by which αvβ6 integrin regulates immune suppression in colon cancer remains largely unexplored.

PD-L1 is a major player in the immune system that can facilitate evasion of immune-mediated control of cancer cell growth. High expression of PD-L1 in tumor cells and other cell types in the tumor microenvironment leads to the binding of PD-1 by PD-L1, resulting in the exhaustion of T cells, thus attenuating tumor-specific immunity and promoting tumor progression [24, 25]. For colon cancer, PD-L1 has been identified as a possible target for immunotherapy. In this study, by analyzing bioinformatics data from the TGCA database and clinical colon cancer specimens, we demonstrated that the expression of αvβ6 was significantly associated with CD8+ T lymphocyte density in colon cancer and that αvβ6 expression was significantly correlated with PD-L1 expression. PD-L1 expression may be constitutive or regulated by numerous signaling pathways that are stimulated in the tumor microenvironment. Moreover, tumor cell-intrinsic mechanisms are emerging as novel agents that can be targeted to mediate PD-L1 expression and tumor immune evasion [26]. Therefore, since αvβ6 on tumor cells mediates cell-cell and cell-matrix interactions through signaling and plays numerous critical roles in the tumor microenvironment, we speculate that αvβ6 may be involved in the regulation of PD-L1 expression and antitumor immunity in colon cancer. In the present study, we focused on the potential role of integrin αvβ6 in the regulation of PD-L1 expression; integrin αvβ6 was found to be involved in tumor immune evasion and the response to anti-PD-1 immunotherapy in colon cancer.

It has been reported that some αv integrins, such as αvβ3, αvβ6 and αvβ8, play multiple and significant roles in tumor immune evasion, some of which are PD-L1-dependent and some of which are PD-L1 independent. Moreover, integrin αvβ3 was found to be involved in the regulation of PD-L1 expression, which was dependent on IFN-γ signaling [27]. In our study, αvβ6 depletion or blockade dramatically decreased PD-L1 expression in HT-29 and WiDr colon cancer cells. In addition, PD-L1 expression induced by IFN-γ was also obviously attenuated by the inhibition of αvβ6. Thus, it is plausible that αvβ6 might regulate the constitutive expression of PD-L1, which likely occurs through molecules usually regulated by (rather than dependent on) IFN-γ signaling.

The β6 subunit contains three domains, the extracellular domain, the transmembrane domain and the endocellular domain, which are essential for binding to many cellular signaling molecules and other proteins. Different parts of integrin αvβ6 have different functions, leading to its multiple regulatory functions in oncogenesis [10, 28]. For example, the extracellular and transmembrane domains are involved in TGF-β activation, adhesion, and epithelial mesenchymal transition (EMT), whereas the cytoplasmic domain affects proliferation, MMP production, migration, and survival [29-31]. It has been demonstrated that heterologous expression of integrin αvβ6 augments cell proliferation, which is mediated by a unique region of the cytoplasmic domain of the β6 subunit [17, 32]. We speculate that the involvement of αvβ6 in the regulation of PD-L1 expression and tumor immune evasion in colon cancer may depend on the αvβ6 downstream signaling axis, which is also associated with its cytoplasmic domain [16, 17, 22]. Notably, we previously revealed a direct link between ERK2 and the cytoplasmic domain of β6 in colon cancer cells, and the increase in ERK/MAPK pathway activity upon transcriptional regulation largely accounted for this direct binding. Nevertheless, while integrin αvβ6 itself has no phosphorylating ability, when nonphosphorylated ERK is bound to the cytoplasmic domain of β6, ERK can be more efficiently phosphorylated by MEK because of conformational changes. The interaction between ERK2 and integrin αvβ6 leads to the phosphorylation of ERK and the activation of various downstream signaling pathways associated with the progression of tumorigenesis [15, 33].

In the current study, we demonstrated that the inhibition of ERK phosphorylation by PD98059 significantly reduced αvβ6-induced PD-L1 expression in colon cancer cells, while PD-L1 expression, which was inhibited by β6-siRNA, was obviously augmented upon treatment with an ERK activating agonist (PMA), which indicated that the ERK pathway was involved in this process. Furthermore, we investigated the effect of direct binding of β6-ERK2 on PD-L1 expression. As expected, our present data confirmed that αvβ6-induced ERK activation resulted in an increase in PD-L1 expression, which was dependent on direct β6-ERK2 binding. Several previous studies have reported that IFN-γ can upregulate PD-L1 expression

through the JAK/STAT1 pathway in malignant tumors [34]. However, we revealed in this study that both β6-siRNA and PD98059 could suppress IFN-γ-induced PD-L1 expression by decreasing ERK1/2 activation rather than by decreasing STAT1 activation. In conclusion, it could be inferred that αvβ6 regulates constitutive and IFN-γ-induced PD-L1 expression in colon cancer cells through activation of the ERK/MAPK pathway, which depends on its direct binding to ERK2. This provides a unifying mechanism for αvβ6-regulated immune evasion in colon cancer.

In the in vivo experiment, we found that αvβ6 promoted tumor growth in an MC38 colon cancer model. Additionally, the positive regulation of PD-L1 expression upon αvβ6 augmentation also occurred in murine tumor cells, which was consistent with our previous in vitro data. The promotion of primary tumor growth is likely a multifactorial effect in which immune dysregulation and PD-L1 variation play major roles. Therefore, we speculate that αvβ6-stimulated tumor growth in colon cancer may be attributed to evasion of antitumor immunity, which depends on upregulated PD-L1 expression. CD8+ T cells are the key immune cells for killing cancer cells harboring major histocompatibility complex (MHC) class I molecules and are the main immune cell types affected by the PD-L1 immunosuppressive checkpoint pathway. PD-L1, which is mainly expressed by antigen-presenting cells (APCs) such as dendritic cells (DCs) and macrophages, as well as tumor cells within the tumor microenvironment, stimulates tumor-specific CD8+ T cell apoptosis, triggering immune escape mechanisms in the tumor [35, 36]. We investigated the immune status of the αvβ6-overexpressing MC38 colon cancer model. As expected, αvβ6 significantly suppressed the antitumor immune effects by decreasing CD8+ T cell infiltration and granzyme B expression in CD8+ T cells. Therefore, these data suggested that αvβ6 was able to induce colon cancer immune evasion by upregulating PD-L1 expression. Although PD-L1 could also suppress CD4+ T cells in the tumor microenvironment, no significant differences in CD4+ T cells were found between the MC38β6 and MC38WT groups. Nevertheless, treatment with an anti-PD-1 antibody increased the infiltration of CD4+ T cells in the MC38β6 groups. These results could be explained in two ways. First, unlike CD8+ T cell depletion, CD4+ T cell depletion in the tumor microenvironment might be mainly associated with PD-L1 expression by tumor-infiltrating antigen-presenting cells (APCs), such as DCs and macrophages, rather than by PD-L1 expression in tumor cells. Second, it was recently reported that anti-PD-1 monotherapy itself promotes increased tumor cell TGF-β signaling and affects the Treg/Th CD4+ T-cell balance, consequently limiting the efficacy of this checkpoint blockade drug. The combination of anti-TGF-β with anti-PD-1 was able to stimulate antitumor immunity and alleviate anti-PD-1 resistance in combination with high levels of intertumoral CD4+ T cells [37]. Activation of TGF-β requires the binding of αv integrin to an RGD peptide present in the LAP, which is held in the extracellular matrix by latent TGF-β-binding proteins. Among the αv integrins, the integrin β6 and β8 phenotypes are particularly important for the activation of TGF-β in vivo [38]. Therefore, the activation of TGF-β by αvβ6 is potentially involved in the regulation of intertumoral CD4+ T cells under anti-PD-1 therapy. Nonetheless, further research is needed to explore the mechanism underlying the role of αvβ6 in regulating CD4+ T cells during ani-PD-1 therapy.

Interestingly, IFN-γ expression in CD8+ T cells in the colon cancer model was significantly increased by αvβ6. Because PD-L1 in tumor cells is generally mediated by IFN-γ signaling, there may be a positive feedback mechanism involved in αvβ6-mediated immune evasion of colon cancer: αvβ6 could upregulate the expression of PD-L1 in colon cancer cells, leading to suppression of the CD8+ T cell immune response in the tumor microenvironment but increasing the release of IFN-γ, which could in turn act on tumor cells to further promote the expression of PD-L1 (**Figure 6**). Accordingly, we conclude that αvβ6 might be a driver of the immune evasion system of colon cancer tumors.

The main limitation of immune checkpoint therapy in colon cancer is that susceptibility is limited to some cancer classifications, such as microsatellite instability (MSI), meaning that only a fraction of patients respond and severe adverse effects develop in the responders [39]. To improve immunotherapy efficacy, ongoing efforts are aiming to support a “hot” tumor phe-

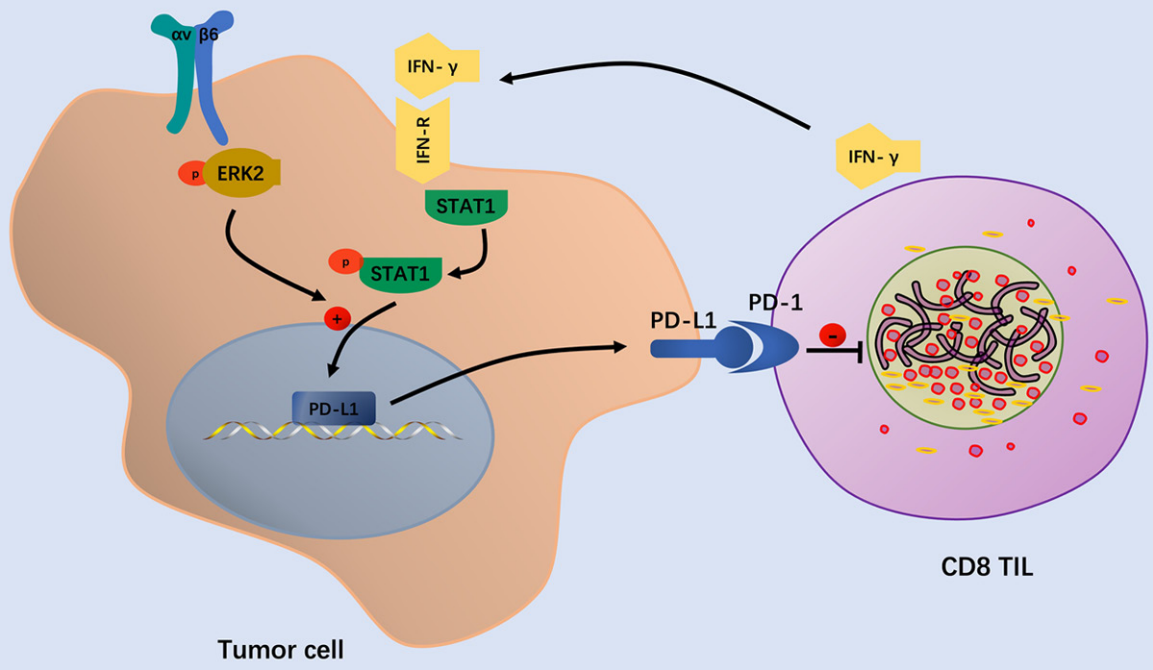

Figure 6. Schematic of the role of integrin αvβ6 in PD-L1 expression and immune escape in colon cancer. αvβ6 regulates PD-L1 expression in colon cancer cells through activation of the ERK/MAPK pathway, which results in suppression of the CD8+ T cells immune response in the tumor microenvironment. However, these factors also increase the release of IFN-γ by CD8+ T cells, which could in turn act on tumor cells to further promote the expression of PD-L1.

notype in the immunosuppressive tumor microenvironment, albeit at the cost of increasing PD-L1 expression, and to combine treatments with checkpoint inhibitors (CPIs).

In this study, we investigated the role of αvβ6 in the regulation of PD-L1 expression and in the tumor immune evasion of colon cancer, and we examined the effects of αvβ6 on anti-PD-1 treatment in colon cancer. Our presented data demonstrate that upregulated αvβ6 expression in colon cancer results in a decreased response to anti-PD-1 immunotherapy. Accordingly, it could be further deduced that αvβ6 blockade might be a potential immunotherapy method and could increase the probability of success of anti-PD-1/PD-L1 blockade and decrease the incidence of adverse effects and resistance. Collectively, these results indicate that as tumor cell-intrinsic mechanisms are emerging, and novel targets and players mediating a response to anti-PD-1/PD-L1 immunotherapies are revealed; integrin αvβ6 may represent a new biomarker for patient selection for immunotherapy with anti-PD-1/PD-L1 in colon cancer and is a potential novel target for the immunotherapy of colon cancer patients.

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Disclosure of conflict of interest

None.

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References

[1] Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A and Bray F. Global cancer statistics 2020: GLOBOCAN estimates of

incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2021; 71: 209-249.

[2] Kopetz S, Chang GJ, Overman MJ, Eng C, Sargent DJ, Larson DW, Grothey A, Vauthey JN, Nagorney DM and McWilliams RR. Improved survival in metastatic colorectal cancer is associated with adoption of hepatic resection and improved chemotherapy. J Clin Oncol 2009; 27: 3677-3683.

[3] Chalabi M, Fanchi LF, Dijkstra KK, Van den Berg JG, Aalbers AG, Sikorska K, Lopez-Yurda M, Grootscholten C, Beets GL, Snaebjornsson P, Maas M, Mertz M, Veninga V, Bounova G, Broeks A, Beets-Tan RG, de Wijkerslooth TR, van Lent AU, Marsman HA, Nuijten E, Kok NF, Kuiper M, Verbeek WH, Kok M, Van Leerdam ME, Schumacher TN, Voest EE and Haanen JB. Neoadjuvant immunotherapy leads to pathological responses in MMR-proficient and MMR-deficient early-stage colon cancers. Nat Med 2020; 26: 566-576.

[4] Li Y, Liang L, Dai W, Cai G, Xu Y, Li X, Li Q and Cai S. Prognostic impact of programed cell death-1 (PD-1) and PD-ligand 1 (PD-L1) expression in cancer cells and tumor infiltrating lymphocytes in colorectal cancer. Mol Cancer 2016; 15: 55.

[5] Jiang X, Wang J, Deng X, Xiong F, Ge J, Xiang B, Wu X, Ma J, Zhou M, Li X, Li Y, Li G, Xiong W, Guo C and Zeng Z. Role of the tumor microenvironment in PD-L1/PD-1-mediated tumor immune escape. Mol Cancer 2019; 18: 10.

[6] Bagchi S, Yuan R and Engleman EG. Immune checkpoint inhibitors for the treatment of cancer: clinical impact and mechanisms of response and resistance. Annu Rev Pathol 2021; 16: 223-249.

[7] Yang Z, Wu G, Zhang X, Gao J, Meng C, Liu Y, Wei Q, Sun L, Wei P, Bai Z, Yao H and Zhang Z. Current progress and future perspectives of neoadjuvant anti-PD-1/PD-L1 therapy for colorectal cancer. Front Immunol 2022; 13: 1001444.

[8] Marzagalli M, Ebelt ND and Manuel ER. Unraveling the crosstalk between melanoma and immune cells in the tumor microenvironment. Semin Cancer Biol 2019; 59: 236-250.

[9] Lei X, Lei Y, Li JK, Du WX, Li RG, Yang J, Li J, Li F and Tan HB. Immune cells within the tumor microenvironment: biological functions and roles in cancer immunotherapy. Cancer Lett 2020; 470: 126-133.

[10] Hynes RO. Integrins: bidirectional, allosteric signaling machines. Cell 2002; 110: 673-687.

[11] Brucher BL and Jamall IS. Cell-cell communication in the tumor microenvironment, carcinogenesis, and anticancer treatment. Cell Physiol Biochem 2014; 34: 213-243.

[12] Hood JD and Cheresh DA. Role of integrins in cell invasion and migration. Nat Rev Cancer 2002; 2: 91-100.

[13] Bates RC, Bellovin DI, Brown C, Maynard E, Wu B, Kawakatsu H, Sheppard D, Oettgen P and Mercurio AM. Transcriptional activation of integrin beta6 during the epithelial-mesenchymal transition defines a novel prognostic indicator of aggressive colon carcinoma. J Clin Invest 2005; 115: 339-347.

[14] Niu J and Li Z. The roles of integrin alphavbeta6 in cancer. Cancer Lett 2017; 403: 128-137.

[15] Gao H, Peng C, Liang B, Shahbaz M, Liu S, Wang B, Sun Q, Niu Z, Niu W, Liu E, Wang J, Lin P, Wang J and Niu J. beta6 integrin induces the expression of metalloproteinase-3 and metalloproteinase-9 in colon cancer cells via ERK-ETS1 pathway. Cancer Lett 2014; 354: 427-437.

[16] Niu J, Dorahy DJ, Gu X, Scott RJ, Draganic B, Ahmed N and Agrez MV. Integrin expression in colon cancer cells is regulated by the cytoplasmic domain of the beta6 integrin subunit. Int J Cancer 2002; 99: 529-537.

[17] Ahmed N, Niu J, Dorahy DJ, Gu X, Andrews S, Meldrum CJ, Scott RJ, Baker MS, Macreadie IG and Agrez MV. Direct integrin alphavbeta6-ERK binding: implications for tumour growth. Oncogene 2002; 21: 1370-1380.

[18] Dodagatta-Marri E, Ma HY, Liang B, Li J, Meyer DS, Chen SY, Sun KH, Ren X, Zivak B, Rosenblum MD, Headley MB, Pinzas L, Reed NI, Del Cid JS, Hann BC, Yang S, Giddabasappa A, Noorbehesht K, Yang B, Dal Porto J, Tsukui T, Niessen K, Atakilit A, Akhurst RJ and Sheppard D. Integrin alphavbeta8 on T cells suppresses anti-tumor immunity in multiple models and is a promising target for tumor immunotherapy. Cell Rep 2021; 36: 109309.

[19] Yang SB, Du Y, Wu BY, Xu SP, Wen JB, Zhu M, Cai CH and Yang PC. Integrin alphavbeta6 promotes tumor tolerance in colorectal cancer. Cancer Immunol Immunother 2012; 61: 335-342.

[20] Napolitano S, Matrone N, Muddassir AL, Martini G, Sorokin A, De Falco V, Giunta EF, Ciardiello D, Martinelli E, Belli V, Furia M, Kopetz S, Morgillo F, Ciardiello F and Troiani T. Triple blockade of EGFR, MEK and PD-L1 has antitumor activity in colorectal cancer models with constitutive activation of MAPK signaling and PD-L1 overexpression. J Exp Clin Cancer Res 2019; 38: 492.

[21] Falcinelli M, Al-Hity G, Baron S, Mampay M, Allen MC, Samuels M, Jones W, Cilibrasi C, Flaherty RL, Giamas G, Thaker PH and Flint MS. Propranolol reduces IFN-gamma driven PD-L1 immunosuppression and improves anti-tu-

mour immunity in ovarian cancer. Brain Behav Immun 2023; 110: 1-12.
[22] Gu X, Niu J, Dorahy DJ, Scott R and Agrez MV. Integrin alpha(v)beta6-associated ERK2 mediates MMP-9 secretion in colon cancer cells. Br J Cancer 2002; 87: 348-351.
[23] Liang B, Li L, Miao R, Wang J, Chen Y, Li Z, Zou X and Zhou M. Expression of interleukin-6 and integrin alphanubeta6 in colon cancer: association with clinical outcomes and prognostic implications. Cancer Invest 2019; 37: 174-184.
[24] Li C, Jiang P, Wei S, Xu X and Wang J. Regulatory T cells in tumor microenvironment: new mechanisms, potential therapeutic strategies and future prospects. Mol Cancer 2020; 19: 116.
[25] Yao H, Lan J, Li C, Shi H, Brosseau JP, Wang H, Lu H, Fang C, Zhang Y, Liang L, Zhou X, Wang C, Xue Y, Cui Y and Xu J. Inhibiting PD-L1 palmitoylation enhances T-cell immune responses against tumours. Nat Biomed Eng 2019; 3: 306-317.
[26] Yi M, Niu M, Xu L, Luo S and Wu K. Regulation of PD-L1 expression in the tumor microenvironment. J Hematol Oncol 2021; 14: 10.
[27] Vannini A, Leoni V, Barboni C, Sanapo M, Zaghini A, Malatesta P, Campadelli-Fiume G and Gianni T. alphavbeta3-integrin regulates PD-L1 expression and is involved in cancer immune evasion. Proc Natl Acad Sci U S A 2019; 116: 20141-20150.
[28] Fernandez-Ruiz E and Sanchez-Madrid F. Regional localization of the human integrin beta 6 gene (ITGB6) to chromosome 2q24-q31. Genomics 1994; 21: 638-640.
[29] Thomas GJ, Hart IR, Speight PM and Marshall JF. Binding of TGF-beta1 latency-associated peptide (LAP) to alpha(v)beta6 integrin modulates behaviour of squamous carcinoma cells. Br J Cancer 2002; 87: 859-867.
[30] Munger JS, Huang X, Kawakatsu H, Griffiths MJ, Dalton SL, Wu J, Pittet JF, Kaminski N, Garat C, Matthay MA, Rifkin DB and Sheppard D. The integrin alpha v beta 6 binds and activates latent TGF beta 1: a mechanism for regulating pulmonary inflammation and fibrosis. Cell 1999; 96: 319-328.
[31] Fontana L, Chen Y, Prijatelj P, Sakai T, Fassler R, Sakai LY and Rifkin DB. Fibronectin is required for integrin alphavbeta6-mediated activation of latent TGF-beta complexes containing LTBP-1. FASEB J 2005; 19: 1798-1808.
[32] Yokosaki Y, Monis H, Chen J and Sheppard D. Differential effects of the integrins alpha9beta1, alphavbeta3, and alphavbeta6 on cell proliferative responses to tenascin. Roles of the beta subunit extracellular and cytoplasmic domains. J Biol Chem 1996; 271: 24144-24150.
[33] Wang J, Wu J, Hong J, Chen R, Xu K, Niu W, Peng C, Liu E, Wang J, Liu S, Agrez M and Niu J. PKC promotes the migration of colon cancer cells by regulating the internalization and recycling of integrin alphavbeta6. Cancer Lett 2011; 311: 38-47.
[34] Zhao L, Zhang W, Luan F, Chen X, Wu H, He Q, Weng Q, Ding L and Yang B. Butein suppresses PD-L1 expression via downregulating STAT1 in non-small cell lung cancer. Biomed Pharmacother 2023; 157: 114030.
[35] Schutz F, Stefanovic S, Mayer L, von Au A, Domschke C and Sohn C. PD-1/PD-L1 pathway in breast cancer. Oncol Res Treat 2017; 40: 294-297.
[36] Ai L, Xu A and Xu J. Roles of PD-1/PD-L1 pathway: signaling, cancer, and beyond. Adv Exp Med Biol 2020; 1248: 33-59.
[37] Dodagatta-Marri E, Meyer DS, Reeves MQ, Paniagua R, To MD, Binnewies M, Broz ML, Mori H, Wu D, Adoumie M, Del Rosario R, Li O, Buchmann T, Liang B, Malato J, Arce Vargus F, Sheppard D, Hann BC, Mirza A, Quezada SA, Rosenblum MD, Krummel MF, Balmain A and Akhurst RJ. alpha-PD-1 therapy elevates Treg/Th balance and increases tumor cell pSmad3 that are both targeted by alpha-TGFbeta antibody to promote durable rejection and immunity in squamous cell carcinomas. J Immunother Cancer 2019; 7: 62.
[38] Shi M, Zhu J, Wang R, Chen X, Mi L, Walz T and Springer TA. Latent TGF-beta structure and activation. Nature 2011; 474: 343-349.
[39] Yi M, Jiao D, Xu H, Liu Q, Zhao W, Han X and Wu K. Biomarkers for predicting efficacy of PD-1/PD-L1 inhibitors. Mol Cancer 2018; 17: 129.