ORIGINAL ARTICLE

Tumor-derived immunoglobulin like transcript 5 induces suppressive immunocyte infiltration in colorectal cancer

Wenjing Shi[1] | Fang Zhang[2] | Xiaozheng Chen[3] | Shuyun Wang[4] | Haiqin Zhang[2] | Zijiang Yang[1] | Guiying Wang[5] | Yan Zheng[6] | Yali Han[7] | Yuping Sun[4] | Aiqin Gao[8]

[1]Jinan Central Hospital, Shandong University, Jinan, Shandong, China

[2]Department of Oncology, Jinan Central Hospital Affiliated to Shandong First Medical University, Jinan, Shandong, China

[3]Shandong Cancer Hospital and Institute, Shandong Academy of Medical Sciences, Shandong First Medical University, Jinan, Shandong, China

[4]Phase I Clinical Research Center, Shandong Cancer Hospital and Institute, Shandong Academy of Medical Sciences, Shandong First Medical University, Jinan, Shandong, China

[5]Linqing People's Hospital, Linqing, Shandong, China

[6]Research Center of Translational Medicine, Jinan Central Hospital Affiliated to Shandong First Medical University, Jinan, Shandong, China

[7]Department of Radiation Oncology, Qilu Hospital, Cheeloo College of Medicine, Shandong University, Jinan, China

[8]Department of Thoracic Radiation Oncology, Shandong Cancer Hospital and Institute, Shandong Academy of Medical Sciences, Shandong First Medical University, Jinan, Shandong, China

Correspondence
Yuping Sun, Phase I Clinical Research Center, Shandong Cancer Hospital and Institute, Shandong Academy of Medical Sciences, Shandong First Medical University, 440 Jiyan Road, Jinan, Shandong 250117, China.
Email: 13370582181@163.com

Aiqin Gao, Department of Thoracic Radiation Oncology, Shandong Cancer Hospital and Institute, Shandong Academy of Medical Sciences, Shandong First Medical University, 440 Jiyan Road, Jinan, Shandong, 250117, China.
Email: gaoaiqin032303145@163.com

Abstract

Infiltration of immunosuppressive cells in the tumor microenvironment (TME) induced colorectal cancer (CRC) progression and its resistance to immunotherapy. Identification of tumor-specific factors to modulate inhibitory immunocyte infiltration would provide alternative and novel targets for CRC immunotherapy. Immunoglobulin-like transcript (ILT) 5 is a negative regulator of myeloid cell activation. However, its expression and functional role in solid tumors is still unknown. Using human CRC tissues and cell lines, we found that ILT5 was highly expressed in CRC cells compared with normal colorectal epithelial cells. Enriched ILT5 in tumor cells was correlated with advanced tumor stages and poor patient survival. Our subsequent in vitro and in vivo studies revealed that tumor-derived ILT5 inhibited the infiltration of T cells, especially that of CD8$^+$ T cells in the TME, creating suppressive T-cell contexture. Furthermore, ILT5 directed M2-like polarization of tumor-associated macrophages (TAMs). Inhibition of tumor-derived ILT5 restored the immunosuppressive T-cell and TAM contexture, and restricted CRC progression. Our findings identified ILT5 expression in solid tumor cells for the first time and raised ILT5 as a potential immunotarget and prognostic predictor in CRC.

Wenjing Shi and Fang Zhang contributed equally to this work.

1 | INTRODUCTION

Colorectal cancer (CRC) is the third most common and the second most deadly cancer worldwide, accounting for 1/10 of cancer-related deaths.[1] Since most CRC patients are diagnosed as advanced and incurable disease stages, systemic therapy, including chemotherapy, radiotherapy, and targeted therapy, represents the dominant strategies for CRC treatment.[2] Immunotherapy targeting programmed cell death protein 1 (PD-1)/programmd cell death 1 ligand 1(PD-L1) signaling has changed the paradigm of solid tumor treatment in the last decade.[3] However, little clinical benefit has been observed in CRC patients because of the unique and suppressive immune microenvironment.[4] Hence, there is an urgent need to explore the tumor-regulated immune environment and develop novel and alternative immunotargets.

Immunoglobulin-like transcript (ILT) 5, also known as LILRB3/LIR3/CD85a, is an inhibitory member of the activating and inhibitory immunoglobulin-like transcripts (ILTs) which modulate activation of immune cells.[5,6] ILT5 is mainly expressed in myeloid cells, including monocytes, macrophages, dendritic cells (DC), granulocytes, basophils, and eosinophils.[7,8] The ortholog of ILT5 in mouse is paired immunoglobulin-like receptor B (PIR-B).[9] Ligation of ILT5 can recruit tyrosine phosphatase (SHP-1 or SHIP) containing an Src homology 2 (SH2) domain,[5,6,10] subsequently suppress neutrophil and basophil activation,[11,12] inhibit Th1 cell proliferation and differentiation,[13] induce M2 polarization of monocytes,[14–16] and promote the antigen presentation of DCs.[17] Therefore, ILT5 functions as a negative immune regulator in autoimmune diseases, sepsis, allotransplant tolerance, and HIV infection.[12,13,17,18] However, ILT5 expression in tumor cells and its function in antitumor immunity is still unknown.

T cells and tumor-associated macrophages (TAMs) are the most frequent and important immune cell components harnessing antitumor immune response in the tumor microenvironment (TME).[19,20] T cells are the major contributors and effectors in antitumor immune response. Among the myriad receptors expressed by T cells, CD3 is a unique molecule that is able to convert the presence of specific antigens into the intracellular signals necessary to trigger an immune response to tumors.[21,22] As effector T cells, CD8^{+} T cells recognize antigen CD3 molecules and eliminate tumors mainly by inducing cell death through perforin granzyme and Fas/Fas ligand pathways.[23,24] In addition to T cells, TAMs are another dominant immune cell component in the TME. They often exhibit the M2-like phenotype,[25,26] and have been reported to affect virtually almost every step of tumor cell metastasis, including invasion, vascularization, intravasation, extravasation, establishment of pre-metastatic niches, and maintenance of the circulating tumor cell survival.[27] Therefore, we focused on ILT5-regulated T-cell and macrophage infiltration and phenotypes in the current study.

In our efforts to explore the expression and immunomodulatory function of ILT5 in CRC, we found that ILT5 was highly expressed in CRC cells and was an adverse prognostic biomarker. Tumor-derived ILT5 inhibited the infiltration of T cells, especially that of CD8^{+} T cells, and directed the M2-like polarization of TAMs, creating a suppressive tumor-immune microenvironment (TIME). Inhibition of tumor-derived ILT5 restored the immunosuppressive TME and restricted CRC progression. Our findings identified ILT5 expression in solid tumor cells for the first time and raised ILT5 as a potential immunotarget and prognostic predictor in CRC.

2 | MATERIALS AND METHODS

2.1 | Patients and tissue samples

With the approval of the review committee and the ethics committee, we collected paraffin-embedded tumors and normal tissues from 129 consecutive CRC patients between January 2013 and December 2015. All patients underwent primary surgeries without chemotherapy, radiotherapy or targeted therapy. The demographic and clinicopathological characteristics of patients are shown in Table 1.

2.2 | Immunohistochemical analysis

Immunohistochemical staining was used to detect the expression of ILT5, the infiltration of T-cell and macrophage subsets in paraffin-embedded human and mouse CRC tissues using immunohistochemical staining kit (zsbio; Cat No. SP-9000), as we previously described.[28] The primary and secondary antibodies are listed in Table S1.

2.3 | Evaluation of ILT5 expression and immune cell infiltration by immunohistochemical staining

Each slide was randomly intercepted to five visual fields under a 400× magnification microscope and evaluated by two independent researchers. ILT5 was determined according to the staining intensity and the percentage of positive cells. The staining intensity was defined as 0 = none, 1 = weak, 2 = intermediate, and 3 = strong. The percentage scores of positive cells were 1 (≤25%), 2 (26%–50%), 3 (51%–75%), and 4 (≥76%). The final score of the slide was determined by the product of the two scores. The cut-off scores for low and high

TABLE 1 Correlations between ILT5 expression and clinicopathological parameters in primary CRC patients (n = 129)

Clinicopathological parameters	ILT5 expression		χ^2	p
	Low (n = 61)	High (n = 68)		
T stage				
pT1-2 (n = 10)	8 (80%)	2 (20%)	4.654	**<.05**
pT3-4 (n = 119)	53 (44.5%)	66 (55.5%)		
Lymph node metastasis				
pN0 (n = 73)	39 (53.4%)	34 (46.6%)	6.669	**<.05**
pN1 (n = 30)	8 (26.7%)	22 (73.3%)		
pN2 (n = 26)	14 (53.8%)	12 (46.2%)		
Metastasis (pM)				
pM0 (n = 123)	61 (49.6%)	62 (50.4%)	5.645	**<.05**
pM1 (n = 6)	0 (0.0%)	6 (100.0%)		
TNM stage				
Stage I (n = 8)	7 (87.5%)	1 (12.5%)	10.02	**<.05**
Stage II (n = 63)	32 (50.8%)	31 (49.2%)		
Stage III (n = 54)	22 (40.7%)	32 (59.3%)		
Stage IV (n = 4)	0 (0.0%)	4 (100.0%)		
Differentiation degree				
G1-2 (n = 127)	60 (47.2%)	67 (52.8%)	0.005999	.9383
G3 (n = 2)	1 (50.0%)	1 (50.0%)		
Age (media n = 69)				
≤69 (n = 65)	32 (49.2%)	33 (50.8%)	0.1986	.6558
>69 (n = 64)	29 (45.3%)	35 (54.7%)		
Pathological type				
Mucous adenocarcinoma (n = 14)	7 (50.0%)	7 (50.0%)	0.04638	.8295
No mucous adenocarcinoma (n = 115)	54 (47.0%)	61 (53.0%)		
Tumor size (median = 5 cm)				
≤5 (n = 65)	33 (50.8%)	32 (49.2%)	0.7743	.3789
>5 (n = 64)	28 (43.8%)	36 (56.2%)		
Gender				
Male (n = 86)	40 (46.5%)	46 (53.5%)	0.0622	.8031
Female (n = 43)	21 (48.8%)	22 (51.2%)		
Lymph-vascular space invasion				
Yes (n = 74)	38 (51.4%)	36 (48.6%)	1.150	.2835
No (n = 55)	23 (41.8%)	32 (58.2%)		

Abbreviations: CRC, colorectal cancer; ILT5, immunoglobulin-like transcript 5; M stage, distant metastasis; N stage, lymph node metastasis; pTNM, pathological TNM; T stage, invasion depth. The Significance of bold values was set at P<0.05 Statistical significant.

ILT5 expression were <5 and ≥5, respectively. The number of cell infiltrations was calculated under a unified 400× microscope. The infiltration proportion of T cells was measured by the frequency of $CD3^+$ T cells in total immune cells, and the frequency of different T-cell subsets was calculated by the fraction of $CD4^+$/$CD8^+$/$FOXP3^+$/$IFN-\gamma^+$ T cells in $CD3^+$ T cells. The proportion of M2-like macrophages was calculated by the frequency of $CD163^+$ cells in CD68. The average of T-cell count and the average percentage of M2-like TAMs were selected as the cut-off values of immune cell infiltration.

2.4 | Immunofluorescence staining of ILT5 in CRC tissues

The expression of ILT5 in human CRC tissues was detected by immunofluorescence staining, as previously described.[29] The antibodies used are shown in Table S1. The expression level of ILT5 was scored as 0 to 3 according to different fluorescence intensities, which were defined as 0 = none, 1 = weak, 2 = intermediate, and 3 = strong.

(A) Weak / Intermediate / Strong — ILT5 (positive) / PBS (negative)

(B) Adjacent normal tissues / Cancer tissues

(C) ILT5 IHC scores — Adjacent normal tissues / Cancer tissues ****

(D) ILT5 / DAPI / MERGE — Adjacent normal tissues / Cancer tissues

(E) ILT5 IF indensity — Adjacent normal tissues / Cancer tissues **

(F) GSE33113 *** — Normal (n = 6) / Colon tumor (n = 90); GSE87211 **** — Normal (n = 160) / Rectal tumor (n = 203)

(G) Relative ILT5 mRNA expression — CCD-18Co, NCM460 (ns), SW480 (***), SW620 (**), DLD-1 (ns), LOVO (*), HCT116 (ns), HT29 (****)

(H) ILT5 / GAPDH — CCD-18Co, NCM460, SW480, SW620, DLD-1, LOVO, HCT116, HT29

(I) Relative ILT5 protein expression — CCD-18Co, NCM460 (ns), SW480 (**), SW620 (*), DLD-1 (*), LOVO (**), HCT116 (**), HT29 (***)
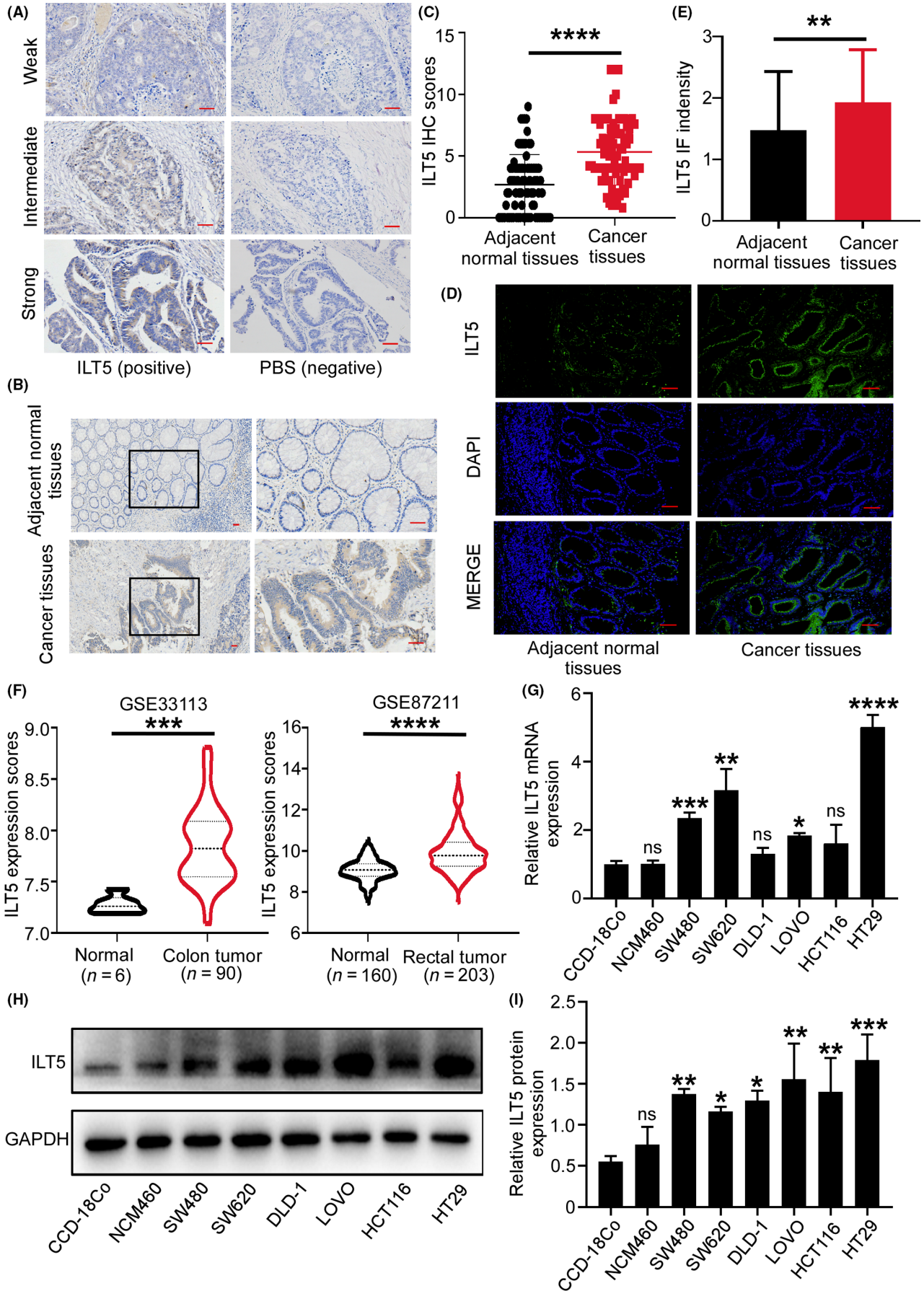
FIGURE 1 Immunoglobulin-like transcript 5 (ILT5) is highly expressed in colorectal cancer (CRC). (A) ILT5 was mainly expressed in the cell membrane and cytoplasm of tumor cells across CRC tissues with different levels. Brown granules were defined as positive staining. Scale bar: 50 μm. (B–E) ILT5 was enriched in the CRC tissues relative to the adjacent normal tissues. (B) Typical images of ILT5 immunohistochemical (IHC) staining in CRC tissues and adjacent normal tissues. Scale bar: 50 μm. (C) IHC statistical results of ILT5 expression in CRC tissues from 129 patients. (D) Typical images of ILT5 expression in CRC tissues by immunofluorescence (IF) analysis. Green fluorescence was defined as positive staining. Scale bar: 50 μm. (E) IF statistical results of ILT5 expression in CRC tissues from 70 patients. (F) Gene Expression Omnibus datasets were used to compare the expression of ILT5 in CRC and corresponding normal tissues, including 90 primary colon tumors and normal colon tissues from six of these patients (GSE33113), 203 primary rectal tumors, and 160 matched mucosa controls (GSE87211). (G–I) Most CRC cell lines showed higher ILT5 expression at both mRNA and protein levels compared with the human colon epithelial cell lines CCD-18Co and NCM460. (G) The gene expression of ILT5 in different human CRC cell lines using real-time quantitative PCR analyses. mRNA levels in each cell line were normalized to the relative quantity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression and then adjusted to the ILT5 level in CCD-18Co cells. The results shown in the histogram are mean ± SD from three independent experiments. (H) The protein levels of ILT5 in different cell lines as determined by western blot. (I) Statistical results of ILT5 expression from three independent western blot analyses. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$
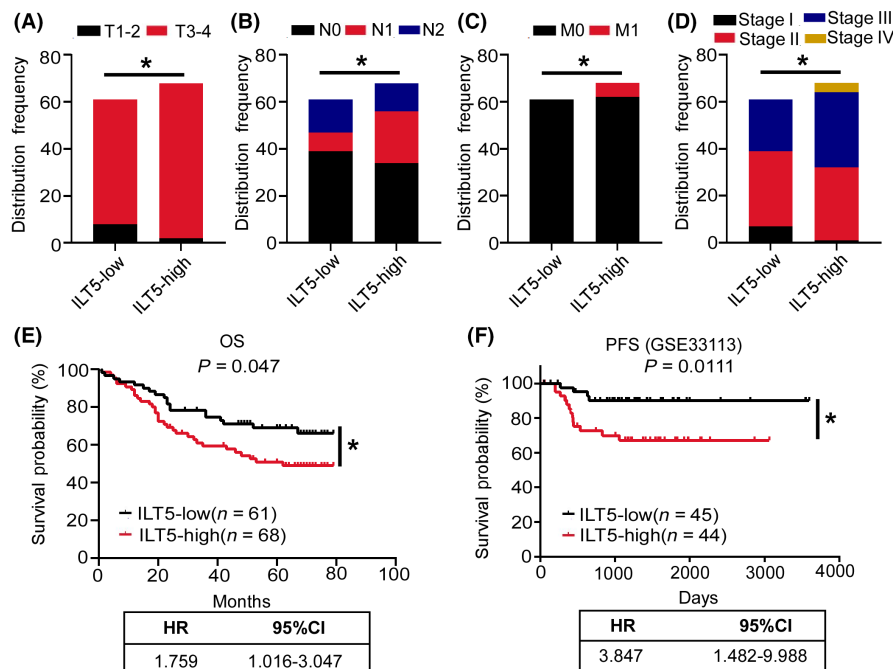

FIGURE 2 Immunoglobulin-like transcript 5 (ILT5) expression in colorectal cancer (CRC) predicts advanced diseases and poor patient survival. (A–D) ILT5 expression was positively correlated with tumor invasion depth, lymph node involvement, distant metastasis, and TNM stages. Compared with patients in the ILT5-low group, those in the ILT5-high group had deeper tumor invasion (A), more regional lymph node involvement (B), more frequent distant metastases (C), and advanced TNM stages (D). Immunohistochemical (IHC) scores of ≥5 and <5 were defined as high and low ILT5 expression, respectively. (E) Patients in the ILT5-low group showed significantly superior overall survival (OS) compared with those in the ILT5-high group in our patient cohort. There were 129 patients in this analysis, and the cut-off scores for ILT5 high and low expression were the same as in (A–D). (F) Low ILT5 expression was correlated with prolonged progression-free survival (PFS) in the GSE33113 dataset. The cut-off value for high and low ILT5 expression was the median. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. HR, hazard ratio; OS, overall survival; PFS, progression-free survival

2.5 | Bioinformatics analysis

Data series GSE33113 and GSE87211 from Gene Expression Omnibus (GEO) datasets were used to analysis the expression of ILT5 in colorectal cancers and corresponding normal tissues. Patient clinical data from GSE33113 was used for progression-free survival (PFS) analysis based on ILT5 expression. The Tumor Immune Estimation Resource (TIMER2.0) online tool and GEPIA2 online tool were used to analyze the correlation of ILT5 with the infiltration level of M2-like macrophage.[30]

2.6 | Tumor cell lines and PIR-B overexpression/knockdown

The human CRC cell lines DLD-1, SW620, SW480, HT29, HCT116, and LOVO and mouse CRC cell line MC38 were purchased from the cell resource center of the Chinese Academy of Sciences. Human colon epithelial cell line CCD-18Co and NCM460 were purchased from Procell Life Science & Technology Company and the American Type Culture Collection, respectively. All the cell lines were cultured in RPMI-1640 or Dulbecco's modified Eagle's medium

supplemented with 10% fetal bovine serum. Lentiviruses for paired immunoglobulin-like receptor B (PIR-B) overexpression or knockdown were purchased from Genecopoeia. MC38 was seeded in a six-well plate at an initial concentration of 1×10^5 cells/well. After 24 h, 1 ml of fresh medium containing PIR-B overexpression/knockdown or control lentiviruses (MOI: 5–10) was added to each well. Forty-eight hours after transfection, 2 μg/ml puromycin was added for 5 days to purify PIR-B overexpression/knockdown MC38 cells.
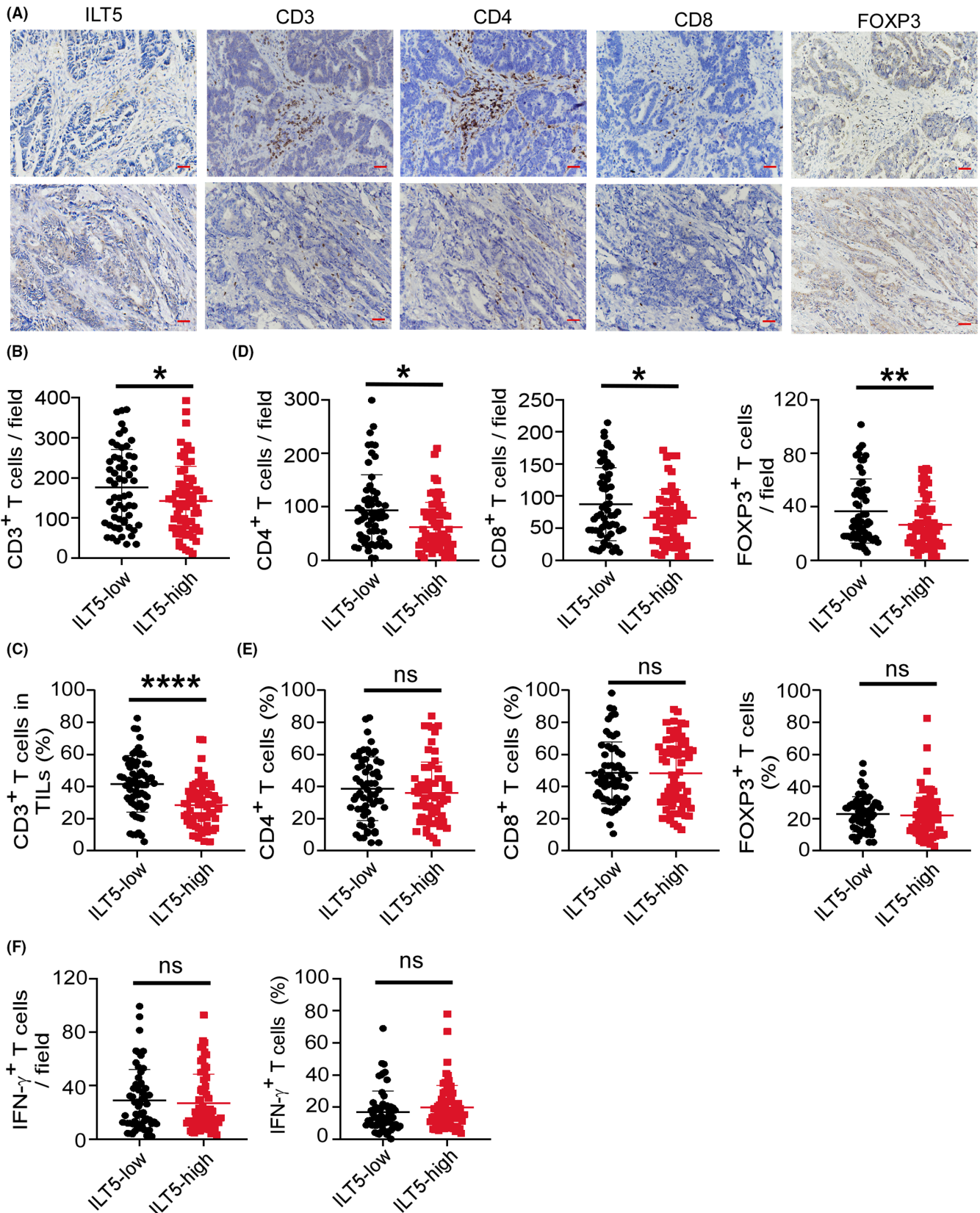
FIGURE 3 Immunoglobulin-like transcript 5 (ILT5) expression is negatively correlated with T-cell infiltration in colorectal cancer (CRC). (A–C) ILT5 expression was negatively correlated with CD3^{+} T-cell infiltration in the tumor microenvironment by immunohistochemical (IHC) analysis. (A) Representative images of ILT5 expression and CD3^{+}/CD4^{+}/CD8^{+}/FOXP3^{+} T-cell infiltration. Each paraffin-embedded tissue was sequentially sectioned for ILT5 and CD3^{+}/CD4^{+}/CD8^{+}/FOXP3^{+} staining. Scale bar: 50 μm. (B, C) Statistical results of CD3^{+} T-cell number (B) and proportion in tumor infiltrating lymphocytes (TILs) (C) from 129 patients. (D, E) The expression of ILT5 was not correlated with the proportion of CD4^{+}/CD8^{+}/FOXP3^{+} T cells in CD3^{+} T cells. (D) All the T-cell subsets, including CD4^{+}, CD8^{+}, and FOXP3^{+} T cells, displayed a significant decrease in ILT5-high patients compared with their ILT5-low counterparts by IHC analysis. (E) The proportions of CD4^{+}, CD8^{+}, and FOXP3^{+} T cells in CD3^{+} T cells were not significantly altered based on ILT5 expression. (F) The expression of ILT5 was not related to IFN-γ levels in T cells. Neither the number nor the proportion of IFN-γ^{+} T cells was different based on ILT5 expression. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$
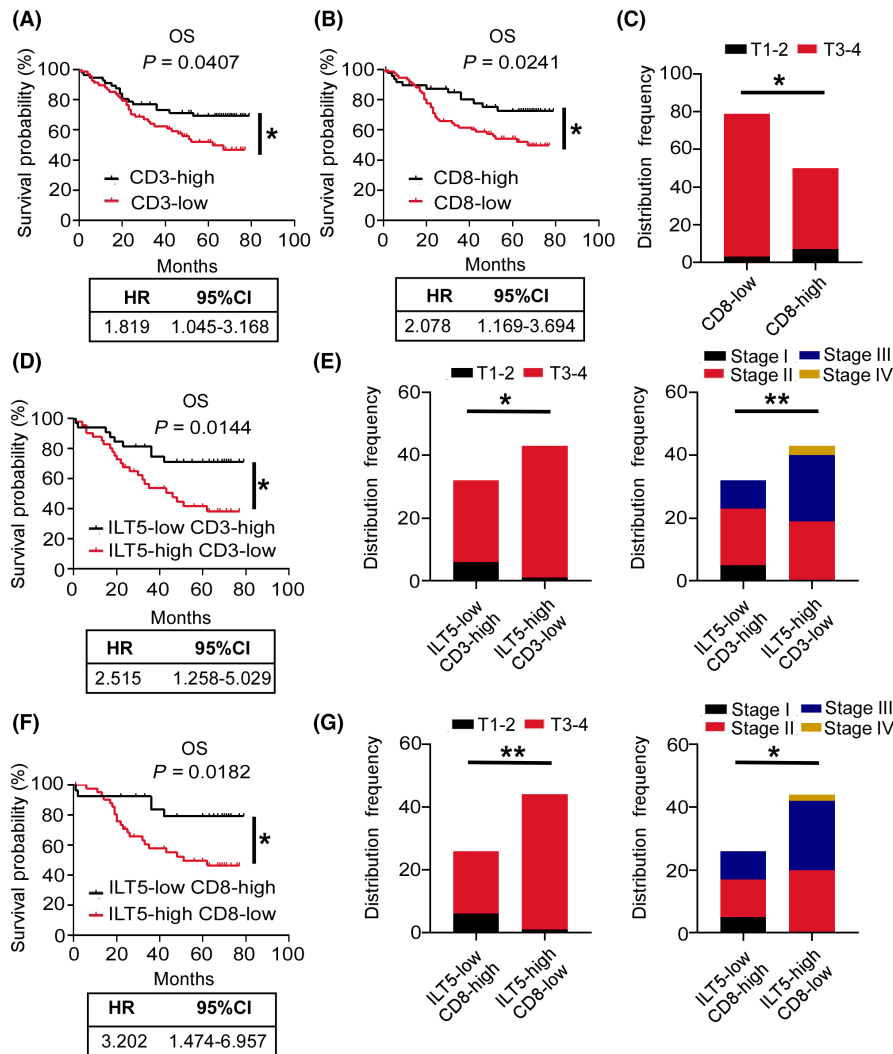

FIGURE 4 High immunoglobulin-like transcript 5 (ILT5) expression in combination with decreased CD3^{+}/CD8^{+} T cells is a stronger indicator for poor patient outcomes. (A, B) Low CD3^{+}/CD8^{+} T-cell infiltration predicts poor patient overall survival (OS). Patients with more CD3^{+}/CD8^{+} T-cell infiltration displayed favorable OS compared with those with low CD3^{+} (A) or CD8^{+} (B) T-cell infiltration. The average infiltrating CD3^{+} or CD8^{+} T-cell number was defined as the cut-off value for high and low infiltration. (C) Patients with low CD8^{+} T-cell density showed deeper tumor invasion compared to those with high CD8^{+} T-cell infiltration. (D) High ILT5 level combined with low CD3^{+} T-cell frequency was a stronger predictor for poor patient OS. Patients in the ILT5-high CD3-low group showed significantly shorter OS than those in the ILT5-low CD3-high group, with a higher hazard ratio (HR) value (2.515) compared with any of the single marker groups (HR = 1.759 for ILT5 alone, HR = 1.819 for CD3^{+} T cell alone). (E) Compared with the patients in the ILT5-low CD3-high group, those in the ILT5-high CD3-low group showed advanced invasion depth and tumor stages. (F) High ILT5 level combined with low CD8^{+}T-cell infiltration was a stronger predictor for poor patient OS. Patients in the ILT5-high CD8-low group showed significantly shorter OS than those in the ILT5-low CD8-high group, with a higher HR (3.202) even than for combined ILT5 and CD3^{+} T-cell infiltration (HR = 2.515). (G) Patients in the ILT5-high CD8-low group showed deeper tumor invasion and advanced tumor stages compared with their counterparts. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. HR, hazard ratio; OS, overall survival; PFS, progression-free survival
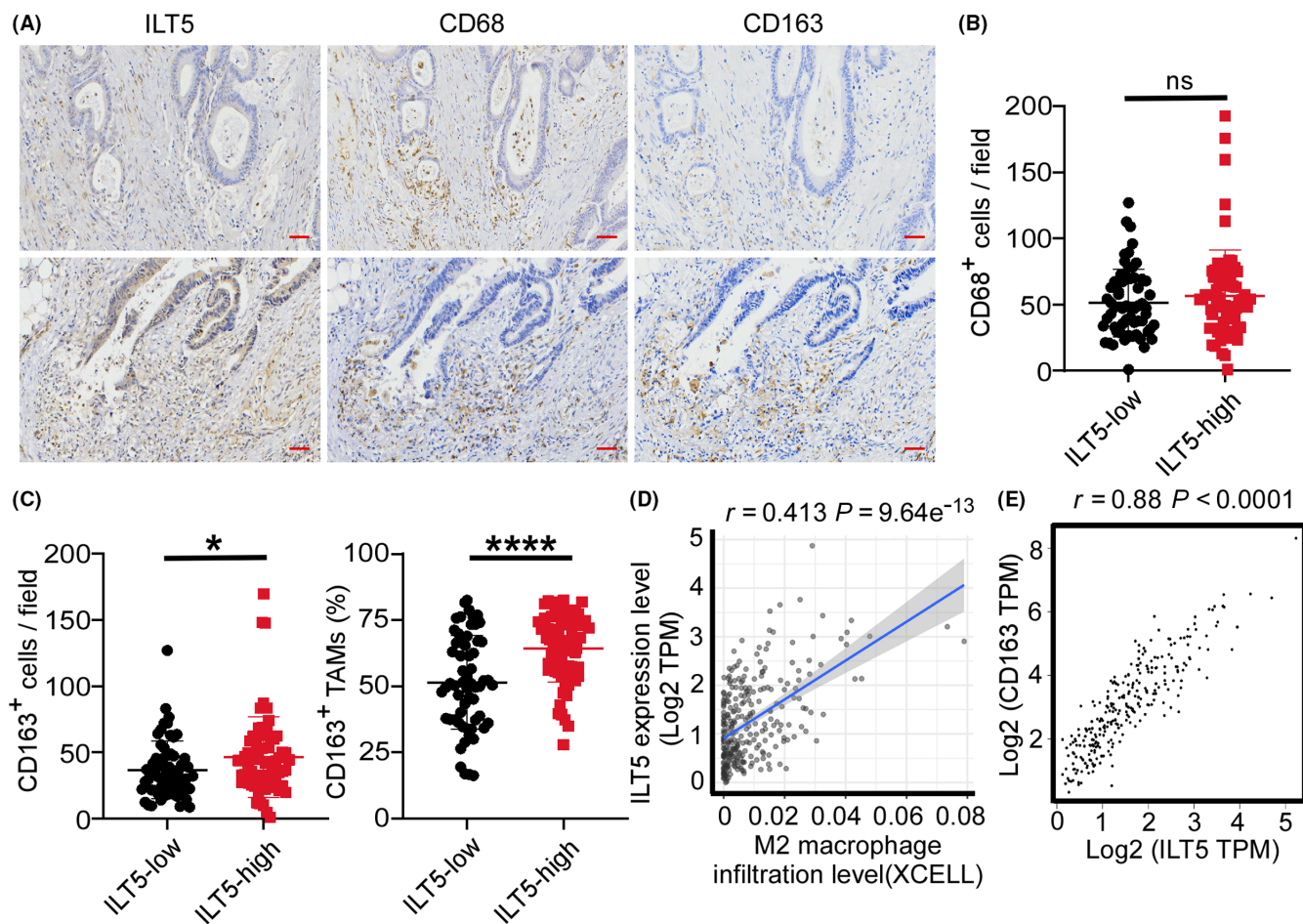
FIGURE 5 Immunoglobulin-like transcript 5 (ILT5) overexpression predicts M2-like polarization of tumor-associated macrophages (TAMs). (A–C) High ILT5 expression indicates increased M2-like (CD163+) TAMs rather than total (CD68+) TAM infiltration. (A) Representative images of ILT5 expression, M2-like, and total TAM infiltration in colorectal cancer. Scale bar: 50 μm. (B) Statistical results for total TAM density in 129 patients based on ILT5 expression. (C) Positive correlation of ILT5 expression with M2-like TAM number and frequency from 129 patients. (D, E) High ILT5 level was positively correlated with M2-like TAM infiltration in public databases. The correlations of ILT5 with the infiltration level of M2-like TAMs (%) were analyzed by the online tools TIMER2.0 (D) and GEPIA2 (E). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$

2.7 | RNA extraction and real-time quantitative PCR

RNA extraction and real-time PCR were performed to analyze ILT5 and PIR-B mRNA expression using our previous protocol.[29] Their expression levels were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The used primers are listed in Table S1.

2.8 | Western blot analysis

Western blot analysis of ILT5 expression in CRC and colonic epithelial cell lines was performed following our previous protocol.[29] The antibodies used are listed in Table S1.

2.9 | Flow cytometric analysis

The markers of mouse monocytes and T cells were determined by flow cytometry after surface staining with specific antibodies

conjugated with different fluorescence. The antibodies are listed in Table S1. The stained cells were analyzed on a FACS Calibur flow cytometer (BD Bioscience) and data were analyzed using FlowJo10 software (Tree Star, Inc.).

2.10 | In vivo study

With the approval of the animal welfare ethics committee of Jinan Central Hospital, female C57BL/6 mice (6–8 weeks old) were purchased from Beijing Viewsolid Biotechnology Company and raised under specific pathogen free (SPF) conditions. Mice were randomly divided into four groups ($n = 5$ mice/group). MC38 cells infected with control lentiviruses or PIR-B overexpression/knockdown lentiviruses were subcutaneously injected into the flanks of mice (1×10^6 cells/mouse). The tumor size was measured every 3 or 4 days, and the tumor volume was calculated as $0.5 \times$ length $\times$ width^2.

When the tumors grew to the size limit (20 mm), the mice were euthanized, and the tumors were separated and weighed. Peripheral blood was isolated to analyze the frequency of immune cell
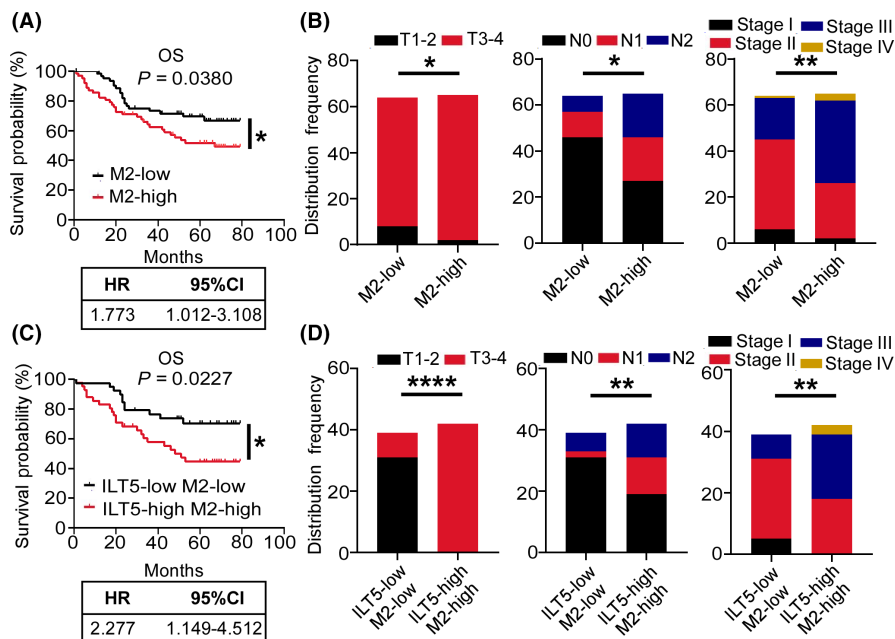
FIGURE 6 Immunoglobulin-like transcript 5 (ILT5)-regulated M2-like polarization of tumor-associated macrophages (TAMs) indicates poor patient outcomes. (A) The infiltration of M2-like (CD163$^+$) TAMs was correlated with poor patient overall survival (OS). The average of infiltrating CD163$^+$ TAMs was used as the cut-off value for high and low M2-like TAMs groups. (B) Patients with high M2-like TAM infiltration displayed deeper tumor invasion depth, more frequent lymph node involvement, and advanced tumor stages compared to those with low M2-like TAMs. (C) High ILT5 in combination with high M2-like TAM density predicted markedly shorter patient OS relative to the ILT5-low M2-low counterpart. Notably, the hazard ratio (HR) value for OS was 2.277, much higher than either of ILT5 (HR = 1.759) or M2-like TAM density (HR = 1.773) alone. (D) Patients with high ILT5 and M2-like TAM infiltration showed more advanced invasion depth, lymph node involvement, and TNM stages than those in the ILT5-low M2-low group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. HR, hazard ratio; OS, overall survival; PFS, progression-free survival

infiltration by flow cytometry. Immunohistochemical (IHC) staining was used to determine the immune cell infiltration in tumor tissues.

2.11 | Survival follow-up and statistical analysis

All patients were followed up to obtain survival data. The last follow-up date was August 20, 2021. Statistical analysis was performed using GraphPad Prism 8.0 software (GraphPad Software Inc.). The correlation of ILT5 expression with clinicopathological features was analyzed using Fisher's exact test, and the intergroup differences were assessed by unpaired Student's t-test. Kaplan–Meier and log-rank tests were used to plot the survival curves. Statistical significance was set at $p < 0.05$.

3 | RESULT

3.1 | ILT5 is highly expressed in colorectal cancer

We collected 129 paraffin-embedded tumor tissues and 70 corresponding adjacent normal tissues from CRC patients, and evaluated ILT5 expression by IHC staining. We found that ILT5 was mainly expressed in the cytoplasm of CRC cells at different levels (Figure 1A). We next compared the difference of ILT5 levels in tumor cells and

adjacent normal epithelial cells. As shown in Figure 1B,C, tumor cells displayed significantly higher ILT5 expression than normal epithelial cells. To further confirm specific ILT5 expression in CRC cells, we used a different ILT5 antibody clone and performed immunofluorescence staining of CRC tissues. As expected, ILT5 expression was markedly higher in CRC cells than in normal epithelial cells (Figure 1D,E). We also determined ILT5 expression in a colon cancer and a rectal cancer dataset of the GEO database. As shown in Figure 1F, both colon and rectal cancer tissues showed significantly improved ILT5 expression compared with corresponding normal tissues. To further verify tumor-specific ILT5 expression, we examined ILT5 in six CRC cell lines and two colorectal epithelial cell lines (CCD-18Co and NCM460) at mRNA and protein levels. We found that the mRNA expression of ILT5 was much higher in most CRC cell lines compared with that in CCD-18Co and NCM460 (Figure 1G). Meanwhile, almost all tumor cell lines showed significantly increased ILT5 protein expression relevant to CCD-18Co and NCM460 by western blot analysis (Figure 1H,I). Collectively, ILT5 is highly expressed in CRC cells.

3.2 | Tumor-derived ILT5 predicts advanced diseases and poor patient survival

To determine the significance of ILT5 expression, we analyzed the clinicopathological characteristics and survival of 129 CRC

patients based on ILT5 levels (Table 1). We found that compared with patients in ILT5-low group, those in the ILT5-high group displayed deeper tumor invasion (Figure 2A), more regional lymph node involvement (Figure 2B), more frequent distant metastasis (Figure 2C), and advanced TNM stages (Figure 2D). The survival analysis revealed that patients in the ILT5-low group had superior overall survival (OS) compared with those in ILT5-high group (Figure 2E). We also analyzed the predictive value of ILT5 on PFS
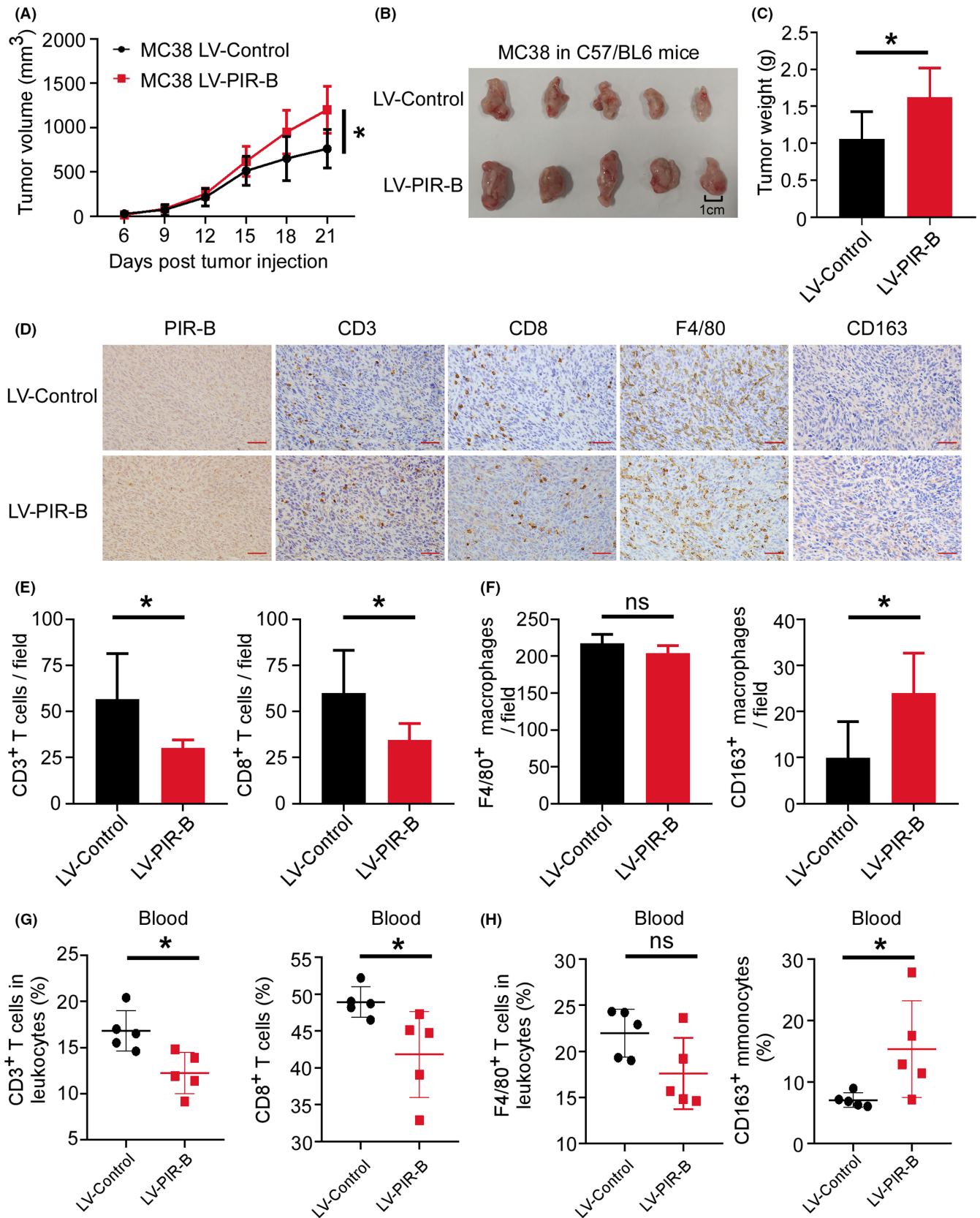
FIGURE 7 Paired immunoglobulin-like receptor B (PIR-B) induces immunosuppressive T-cell/tumor-associated macrophage contexture and tumor growth. (A) PIR-B overexpression significantly promoted the growth of subcutaneous transplanted tumors in C57BL/6 mice. Mouse colon cancer cell line MC38 (1×10^6 cells/mouse) infected with PIR-B overexpression- or control-lentivirus was injected subcutaneously in C57BL/6 mice. Tumor sizes were measured every 3 days and presented as mean ± SD ($n = 5$ mice/group). (B, C) Tumors in the immunoglobulin-like transcript 5 overexpression group displayed remarkably increased sizes and weights compared with those in the control group. Tumor sizes (B) and weights (C) were presented as mean ± SD of each group at the end of the experiment. Scale bar: 1 cm. (D–F) Overexpression of PIR-B inhibited the accumulation of CD3$^+$ and CD8$^+$ T cells in tumor tissues, but increased the density of M2-like macrophages. Consecutive colorectal cancer sections were stained for PIR-B, CD3, CD8, F4/80, and CD163. Representative images of immunohistochemical (IHC) staining for the above molecules are displayed in (D) and the statistical results are displayed in (E) and (F). Scale bar: 50 μm. (G, H) Overexpression of PIR-B significantly reduced the proportion of CD3$^+$ and CD8$^+$ T cells in leukocytes and increased the proportion of CD163$^+$ monocytes. The total leukocytes were calculated as the CD45$^+$ cells. The monocytes were labeled by F4/80 staining. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. LV-Control, MC38 cells transfected with control lentivirus; LV-PIR-B, MC38 cells transfected with PIR-B overexpression lentivirus

using the GEO database. As expected, low ILT5 expression was correlated with prolonged PFS (Figure 2F). Taken together, enriched ILT5 in CRC predicts advanced diseases and poor patient survival.

3.3 | ILT5 expression is negatively correlated with T-cell infiltration

Since T cells are the most important regulator and effectors for tumor eradication, we investigated the correlation of ILT5 expression with T-cell density and subset distribution by IHC. We found that in CRC tissues, high ILT5 expression was correlated with decreased CD3$^+$ T-cell infiltration in the TME (Figure 3A–C). Then we explored ILT5-directed T cell subset distribution by detecting CD4$^+$, CD8$^+$, and FOXP3$^+$ T-cell population. As shown in Figure 3D, the enrichment of ILT5 was negatively correlated with the number of CD4$^+$ T cells, CD8$^+$ T cells, and FOXP3$^+$ T cells in the TME. However, the proportion of these T-cell subsets in total T cells was not significantly different (Figure 3E), suggesting that tumor-derived ILT5 might reduce T-cell recruitment rather than regulate its differentiation. To explore whether ILT5 impacted the killing ability of T cells, we analyzed IFN-γ levels in T cells based on ILT5 levels. Unfortunately, we did not observe a difference in T-cell-derived IFN-γ between the two groups (Figure 3F), suggesting that T-cell function was not markedly altered by ILT5. These findings suggest that ILT5 prevents T-cell infiltration in the TME of CRC, but does not affect T-cell subset distribution and killing ability.

3.4 | High ILT5 expression in combination with decreased CD3$^+$/CD8$^+$ T cells is a stronger indicator for poor patient outcomes

To address the clinical significance of ILT5-orchestrated T-cell infiltration, we determined patient OS and clinicopathological features depending on the infiltration number of different T-cell subsets. We found that patients with high CD3$^+$ T-cell infiltration (CD3-high group) showed beneficial OS compared with their CD3-low counterparts (Figure 4A). We also observed deeper tumor invasion, more

lymph node metastasis, and advanced TNM stage in CD3-low patients compared with the CD3-high group, although without statistical significance (Figure S1A–C). it is likely that low CD8$^+$ T-cell infiltration in the TME is correlated with poor patient OS (Figure 4B), as well as deeper tumor invasion (Figure 4C), advanced lymph node metastasis, and TNM stage (Figure S1D,E). However, we did not observe a significant survival difference based on CD4$^+$ T-cell and FOXP3$^+$ T-cell density (data not shown), suggesting their negligible clinical value.

Next, we analyzed patient survival based on combined ILT5 expression and CD3$^+$ T/CD8$^+$ T-cell infiltration. The patients were classified into four subgroups based on the level of ILT5 expression and infiltrating CD3$^+$ T cells: ILT5-high CD3-high ($n = 25$), ILT5-high CD3-low ($n = 43$), ILT5-low CD3-high ($n = 32$), and ILT5-low CD3-low ($n = 29$). The results showed that patients in the ILT5-low CD3-high group had significant longer OS than those in ILT5-high CD3-low group, with a larger hazard ratio (HR) of OS compared with ILT5 expression or CD3$^+$ T-cell density alone, suggesting that the combined biomarkers are stronger indicators of patient survival (Figure 4D). In addition, the combination of ILT5-high and CD3-low predicted advanced invasive depth and TNM stages (Figure 4E). Similarly, four subgroups were generated based on ILT5 expression and CD8$^+$ T-cell infiltration: ILT5-high CD8-high ($n = 24$), ILT5-high CD8-low ($n = 44$), ILT5-low CD8-high ($n = 26$), and ILT5-low CD8-low ($n = 35$). Patient survival based on ILT5 expression and CD8$^+$ T-cell infiltration showed a similar trend to that of ILT5 and CD3$^+$ T cells (Figure 4F). The ILT5-high CD8-low group also showed advanced invasive depth and TNM stages (Figure 4G). Altogether, ILT5 overexpression in CRC cancer leads to the reduction of CD3$^+$ and CD8$^+$ T-cell infiltration, which are independent predictors of poor patient survival. The combination of CD3$^+$/CD8$^+$ T and ILT5 could better predict the prognosis and pathological stage of patients.

3.5 | ILT5 overexpression predicts M2-like polarization of TAMs

TAMs, usually polarized to M2-like phenotype by TME, are the most frequent and crucial pro-tumoral immune cells in CRC. To probe into ILT5-directed TAM infiltration and subset distribution,
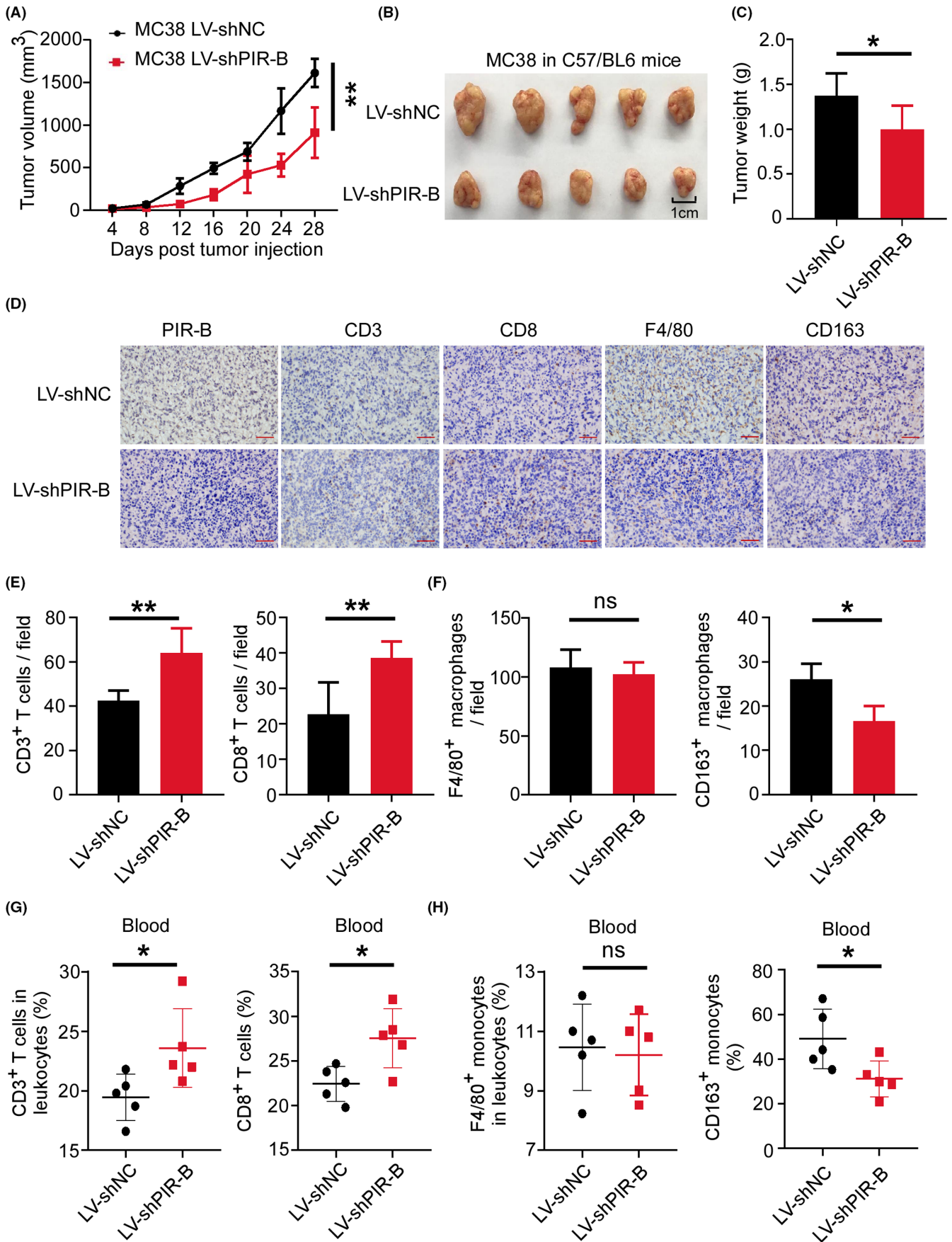
FIGURE 8 Paired immunoglobulin-like receptor B (PIR-B) inhibition prevented the immunosuppressive tumor microenvironment and restricted tumor progression. (A) PIR-B knockdown significantly inhibited the growth of transplanted tumors in C57BL/6 mice. Mouse colon cancer cell line MC38 (1×10^6 cells/mouse) infected with PIR-B knockdown- or control- lentiviruses was subcutaneously inoculated into 6–8-week-old female C57BL/6 mice. Tumor sizes were measured every 4 days and are presented as mean $\pm$ SD ($n = 5$ mice/group). (B, C) PIR-B knockdown in MC38 cells markedly decreased final sizes and weights of transplanted tumors. Tumor sizes (B) and weights (C) are presented as mean $\pm$ SD from each group at the endpoint of the experiments. Scale bar: 1 cm. (D–F) PIR-B inhibition significantly increased the infiltration of CD3$^+$ and CD8$^+$ T cells but reduced that of M2-like tumor-associated macrophages. Consecutive colorectal cancer sections were stained for PIR-B, CD3, CD8, F4/80, and CD163. Representative images of immunohistochemical (IHC) staining for the above molecules are displayed in (D) and the statistical results are displayed in (E) and (F). Scale bar: 50 μm. (G, H) PIR-B knockdown remarkably elevated the percentage of CD3$^+$ and CD8$^+$ T cells in leukocytes (G) but reduced the percentage of CD163$^+$ monocytes in the peripheral blood of tumor-bearing mice by flow cytometry analysis. The total leukocytes were calculated as the CD45$^+$ cells. The monocytes were labeled by F4/80 staining. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. LV-shNC, MC38 cells transfected with control lentivirus; LV-shPIR-B, MC38 cells transfected with PIR-B knockdown lentivirus

we performed IHC staining of CD68 and CD163 in 129 cases of CRC. Although we did not find an obvious association between ILT5 expression and CD68$^+$ macrophage density (Figure 5A,B), we observed remarkably increased density and proportion of M2-like TAMs (CD163$^+$) in the TME of ILT5-high patients compared with the ILT5-low counterpart (Figure 5A,C). Analysis of the TIMER2.0 and GEPIA2 databases yielded similar results showing that ILT5 expression was positively correlated with the infiltration of M2-like TAMs (Figure 5D,E). Altogether, our results indicate that ILT5 expression is positively correlated with the density of M2-like TAMs.

3.6 | ILT5-directed M2-like polarization of TAMs indicates poor patient outcomes

To further explore the significance of ILT5-regulated TAM polarization, we analyzed the clinicopathological parameters and OS in CRC patients depending on M2-like macrophages. The results showed that patients in the M2-high group had poorer OS, more advanced invasion depth, regional lymph node metastasis, and TNM stages (Figure 6A,B), indicating M2-like macrophage as a predictor of poor outcome. In addition, we compared the divergence of clinical characteristics and patient survival based on combined ILT5 expression and M2-like TAM density. As expected, patients with high ILT5 expression as well as enriched M2-like TAMs displayed a poorer OS and a higher probability for advanced invasion depth, regional lymph node metastasis and TNM stages than their ILT5-low M2-low counterparts (Figure 6C,D). These results suggest that high ILT5 expression predicted the M2-like polarization of TAMs, which is an unfavorable indicator for patient outcomes.

3.7 | PIR-B induces immunosuppressive T cell/ TAM contexture and tumor growth.

To verify ILT5-regulated TIME in vivo, we established a CRC transplantation model in C57BL/6 mice using MC38 cells. First, 1×10^6 MC38 cells transfected with PIR-B overexpression or control lentiviruses were subcutaneously injected into the right flank of

C57BL/6 mice. The efficiency of the PIR-B overexpression was determined by real-time PCR and western blot analyses (Figure S2A–C). When the tumors grew to the size limit (length 20 mm), the mice were euthanized, then the tumor and peripheral blood were isolated for subsequent experimentation. We found that PIR-B overexpression accelerated tumor growth relative to the control group (Figure 7A,B). The final tumor weight in each group revealed similar results (Figure 7C). Mouse tumors were wrapped in wax blocks and sectioned for immunohistochemical staining. As shown in Figure 7D–F, tumor tissues with PIR-B overexpression showed significantly decreased CD3$^+$ and CD8$^+$ T-cell infiltration, but increased M2-like TAM densities. We also determined the immunocyte subsets in peripheral blood by flow cytometry analysis. As shown in Figure 7G, PIR-B overexpression decreased the proportion of CD3$^+$ and CD8$^+$ T cells in leukocytes from peripheral blood, but increased CD163$^+$ monocyte frequencies (Figure 7H). These results suggest that ILT5 prevents the infiltration of CD3$^+$ and CD8$^+$ T cells, and induces the M2-like polarization of TAMs, contributing to immunosuppressive TME and CRC growth.

3.8 | PIR-B inhibition prevented immunosuppressive TME and restricted CRC progression

To further clarify the antitumor effect of ILT5 inhibition, we established tumor treatment models in C57BL/6 mice using PIR-B knockdown or control MC38 cells. The efficiency of PIR-B knockdown was determined by real-time PCR and western blot analyses (Figure S2D–F). We found that PIR-B knockdown slowed down the tumor growth compared with that in the control group (Figure 8A–C). The immunohistochemical analysis of final tumor tissues in the two groups showed that PIR-B inhibition increased the infiltration of CD3$^+$ and CD8$^+$ T cells, but decreased M2-like TAM densities (Figure 8D–F). Our flow cytometric analysis of peripheral blood revealed similar results. ILT5 inhibition augmented the frequency of CD3$^+$ and CD8$^+$ T cells, but diminished the fraction of M2-like monocytes in peripheral blood (Figure 8G,H). These results suggest that ILT5 inhibition prevents immunosuppressive TME and potentially represses CRC progression. ILT5 might function as a promising target for CRC immunotherapy.

4 | DISCUSSION

Current immunotherapy targeting PD-1 and PD-L1 pathways has largely changed the paradigm of treatments for most solid tumors. However, only 4%–5% of advanced CRC patients who have deficient mismatch repair (dMMR) or high microsatellite instability (MSI-H) may benefit from immunotherapy due to immunosuppressive TME and insufficient T-cell infiltration.[4,31] Therefore, exploring novel immunotargets to reverse suppressive TME and enhance T-cell infiltration is a potential strategy to develop alternative immunotherapeutics in CRC. Here, we innovatively identified ILT5 expression in CRC cells, delineated its role in manipulating immunosuppressive T-cell and TAM contexture, and proposed ILT5 as a potential immunotarget for CRC therapy. In addition, our in vivo studies indicated that blockage of ILT5 can significantly reverse pro-tumoral TIME and prevent CRC progression.

ILT5 is an immunosuppressive molecule highly expressed in myeloid cells, including monocytes, macrophages, DCs, and granulocytes.[7,8] Previous studies have widely reported its function in sepsis, HIV infection, and rheumatoid arthritis.[13,17,32] Patients with sepsis or HIV infection showed increased ILT5+ immune cells, indicating its immunosuppressive effect.[13,17] However, whether ILT5 is expressed in cancer cells is unknown. Its functional role in modulating antitumor immune response is also unclear. Here, we are the first to report ILT5 expression in solid tumors. In our efforts to address its clinical significance in CRC progression, we found that enriched ILT5 predicted unfavorable survival and advanced tumor stages, raising ILT5 as an adverse prognostic indicator. As a canonical immunosuppressor, we speculated that ILT5 might orchestrate an antitumor immune environment. We focused on the most important immune cell components in the TME, T cells and TAMs, to explore ILT5-regulated TIME. As expected, ILT5 inhibited T-cell infiltration but promoted M2-like TAM accumulation, suggesting the crucial role of ILT5 in creating an inhibitory TIME.

T cells contain heterogeneous subsets either activating or inhibiting immune response, which mainly include CD4+ T cells, CD8+ T cells, and FOXP3+ Treg cells.[33–35] Tumor infiltrates with CD3+ and CD8+ T cells are the immune parameters most consistently and strongly associated with good clinical outcome in CRC.[36] Based on these findings, different scoring systems evaluating lymphocyte infiltrates have been used as useful tools in the prediction of disease progression and efficacy of immunotherapy.[37,38] Consistently, we found the density of CD3+ and CD8+ T cells in CRC indicated favorable patient survival. Meanwhile, we observed that tumor-derived ILT5 restricted CD3+ and CD8+ T-cell infiltration in the TME, partially explaining the mechanism for ILT5-regulated immunosuppression. We also identified decreased CD4+ and FOXP3+ T-cell infiltrates in patients with high ILT5 expression. Unexpectedly, no connection was built between CD4+/FOXP3+ T-cell density and patient outcome based on our data. In recent years, the diversity of CD4+ T-cell form and function has been discovered.[39,40] CD4+ T cells might compose totally opposing subsets with lineage diversity and functional heterogeneity,[41] so the prognostic value of CD4+ T-cell density might depend on the dynamic equilibrium among these different subsets, which is difficult to delineate. Similarly, the prognostic impact of Treg cell infiltration in CRC is also controversial owing to the heterogeneous subgroups which contribute either to progression or to the inhibition of CRC.[42–44] These findings partially support our results. Collectively, our results indicate tumor cell-derived ILT5 is a suppressor of T-cell immunity and mediates the immune escape of CRC.

TAMs are critical immune infiltrates in CRC. In general, they can be classified into two pools, namely, M1- and M2-like TAMs.[45] While M1-like TAMs promote antitumor immune responses by regulating antigen presentation and secreting proinflammatory cytokines,[46] M2-like TAMs promote tumor progression by inducing tumor growth, invasion, vascularization, and immune escape.[47–51] The infiltration of M2-like TAMs in CRC tissue is an adverse prognostic factor for CRC patients.[47,52,53] Our current study also identified the causative links between ILT5 expression and M2-like TAM infiltration, as illustrated by in vitro and in vivo studies. Since the total CD68+ TAMs was not altered by ILT5 modulation, we proposed that ILT5 might harness the polarization of TAMs towards M2-like phenotype, as revealed by high-resolution transcriptome sequencing of human macrophages.[15,16] These results provide another potential mechanism for ILT5-regulated tumor progression.

In summary, ILT5 is enriched in CRC cells, functioning as a negative prognostic biomarker. ILT5 inhibits the infiltration of CD3+ and CD8+ T cells and induces M2-like polarization of TAMs, creating immunosuppressive TME for CRC progression. ILT5 is a potential and novel immunotarget for CRC immunotherapy.

DISCLOSURE

The authors have no conflict of interest to declare. All authors have read the journal's authorship agreement. The manuscript has been reviewed by and approved by all named authors.

ORCID

Wenjing Shi https://orcid.org/0000-0002-3904-5869
Fang Zhang https://orcid.org/0000-0003-1992-5647
Xiaozheng Chen https://orcid.org/0000-0003-2438-2990
Shuyun Wang https://orcid.org/0000-0002-5100-1877
Haiqin Zhang https://orcid.org/0000-0002-5518-480X
Zijiang Yang https://orcid.org/0000-0002-3736-9077
Guiying Wang https://orcid.org/0000-0001-6560-8920
Yan Zheng https://orcid.org/0000-0003-4877-7292

Yali Han https://orcid.org/0000-0003-4533-922X

Yuping Sun https://orcid.org/0000-0002-0804-6850

Aiqin Gao https://orcid.org/0000-0003-1157-6104

REFERENCES

1. Sung H, Ferlay J, Siegel RL, et al. Global Cancer Statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin.* 2021;71(3):209-249.

2. Siegel RL, Miller KD, Goding Sauer A, et al. Colorectal cancer statistics, 2020. *CA Cancer J Clin.* 2020;70(3):145-164.

3. Ribas A, Wolchok JD. Cancer immunotherapy using checkpoint blockade. *Science.* 2018;359(6382):1350-1355.

4. Overman MJ, McDermott R, Leach JL, et al. Nivolumab in patients with metastatic DNA mismatch repair-deficient or microsatellite instability-high colorectal cancer (CheckMate 142): an open-label, multicentre, phase 2 study. *Lancet Oncol.* 2017;18(9):1182-1191.

5. Borges L, Hsu ML, Fanger N, Kubin M, Cosman D. A family of human lymphoid and myeloid Ig-like receptors, some of which bind to MHC class I molecules. *J Immunol.* 1997;159(11):5192-5196.

6. Colonna M, Navarro F, Bellon T, et al. A common inhibitory receptor for major histocompatibility complex class I molecules on human lymphoid and myelomonocytic cells. *J Exp Med.* 1997;186(11):1809-1818.

7. van derTouw W, Chen HM, Pan PY, Chen SH. LILRB receptor-mediated regulation of myeloid cell maturation and function. *Cancer Immunol Immunother.* 2017;66(8):1079-1087.

8. Hudson LE, Allen RL. Leukocyte Ig-like receptors – a model for MHC Class I disease associations. *Front Immunol.* 2016;7:281.

9. Kubagawa H, Cooper MD, Chen CC, et al. Paired immunoglobulin-like receptors of activating and inhibitory types. *Curr Top Microbiol Immunol.* 1999;244:137-149.

10. Nakajima H, Samaridis J, Angman L, Colonna M. Human myeloid cells express an activating ILT receptor (ILT1) that associates with Fc receptor gamma-chain. *J Immunol.* 1999;162(1):5-8.

11. Zhao Y, vanWoudenbergh E, Zhu J, et al. The orphan immune receptor LILRB3 modulates Fc receptor-mediated functions of neutrophils. *J Immunol.* 2020;204(4):954-966.

12. Sloane DE, Tedla N, Awoniyi M, et al. Leukocyte immunoglobulin-like receptors: novel innate receptors for human basophil activation and inhibition. *Blood.* 2004;104(9):2832-2839.

13. Ming S, Li M, Wu M, et al. Immunoglobulin-like transcript 5 inhibits macrophage-mediated bacterial killing and antigen presentation during sepsis. *J Infect Dis.* 2019;220(10):1688-1699.

14. Galvan-Pena S, O'Neill LA. Metabolic reprograming in macrophage polarization. *Front Immunol.* 2014;5:420.

15. Beyer M, Mallmann MR, Xue J, et al. High-resolution transcriptome of human macrophages. *PLoS One.* 2012;7(9):e45466.

16. Martinez FO, Gordon S, Locati M, Mantovani A. Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. *J Immunol.* 2006;177(10):7303-7311.

17. Huang J, Burke PS, Cung TD, et al. Leukocyte immunoglobulin-like receptors maintain unique antigen-presenting properties of circulating myeloid dendritic cells in HIV-1-infected elite controllers. *J Virol.* 2010;84(18):9463-9471.

18. Yeboah M, Papagregoriou C, Jones DC, et al. LILRB3 (ILT5) is a myeloid cell checkpoint that elicits profound immunomodulation. *JCI Insight.* 2020;5(18):e141593.

19. Chanmee T, Ontong P, Konno K, Itano N. Tumor-associated macrophages as major players in the tumor microenvironment. *Cancers (Basel).* 2014;6(3):1670-1690.

20. Bussard KM, Mutkus L, Stumpf K, Gomez-Manzano C, Marini FC. Tumor-associated stromal cells as key contributors to the tumor microenvironment. *Breast Cancer Res.* 2016;18(1):84.

21. Cambier JC. Antigen and Fc receptor signaling. The awesome power of the immunoreceptor tyrosine-based activation motif (ITAM). *J Immunol.* 1995;155(7):3281-3285.

22. Straus DB, Weiss A. The CD3 chains of the T cell antigen receptor associate with the ZAP-70 tyrosine kinase and are tyrosine phosphorylated after receptor stimulation. *J Exp Med.* 1993;178(5):1523-1530.

23. Barry M, Bleackley RC. Cytotoxic T lymphocytes: all roads lead to death. *Nat Rev Immunol.* 2002;2(6):401-409.

24. Golstein P, Griffiths GM. An early history of T cell-mediated cytotoxicity. *Nat Rev Immunol.* 2018;18(8):527-535.

25. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol.* 2002;23(11):549-555.

26. Pollard JW. Tumour-educated macrophages promote tumour progression and metastasis. *Nat Rev Cancer.* 2004;4(1):71-78.

27. Lin Y, Xu J, Lan H. Tumor-associated macrophages in tumor metastasis: biological roles and clinical therapeutic applications. *J Hematol Oncol.* 2019;12(1):76.

28. Li Q, Li J, Wang S, et al. Overexpressed immunoglobulin-like transcript (ILT) 4 in lung adenocarcinoma is correlated with immunosuppressive T cell subset infiltration and poor patient outcomes. *Biomark Res.* 2020;8:11.

29. Chen X, Gao A, Zhang F, et al. ILT4 inhibition prevents TAM- and dysfunctional T cell-mediated immunosuppression and enhances the efficacy of anti-PD-L1 therapy in NSCLC with EGFR activation. *Theranostics.* 2021;11(7):3392-3416.

30. Li T, Fu J, Zeng Z, et al. TIMER2.0 for analysis of tumor-infiltrating immune cells. *Nucleic Acids Res.* 2020;48(W1):W509-W514.

31. Yang Z, Gao A, Shi W, et al. ILT4 in colorectal cancer cells induces suppressive T cell contexture and disease progression. *Onco Targets Ther.* 2021;14:4239-4254.

32. Huynh OA, Hampartzoumian T, Arm JP, et al. Down-regulation of leucocyte immunoglobulin-like receptor expression in the synovium of rheumatoid arthritis patients after treatment with disease-modifying anti-rheumatic drugs. *Rheumatology (Oxford).* 2007;46(5):742-751.

33. Deng G, Song X, Fujimoto S, Piccirillo CA, Nagai Y, Greene MI. Foxp3 post-translational modifications and Treg suppressive activity. *Front Immunol.* 2019;10:2486.

34. Hirahara K, Nakayama T. CD4+ T-cell subsets in inflammatory diseases: beyond the Th1/Th2 paradigm. *Int Immunol.* 2016;28(4):163-171.

35. Stemberger C, Neuenhahn M, Buchholz VR, Busch DH. Origin of CD8+ effector and memory T cell subsets. *Cell Mol Immunol.* 2007;4(6):399-405.

36. Malka D, Lievre A, Andre T, Taieb J, Ducreux M, Bibeau F. Immune scores in colorectal cancer: where are we?*Eur J Cancer.* 2020;140:105-118.

37. Anitei MG, Zeitoun G, Mlecnik B, et al. Prognostic and predictive values of the immunoscore in patients with rectal cancer. *Clin Cancer Res.* 2014;20(7):1891-1899.

38. Pages F, Mlecnik B, Marliot F, et al. International validation of the consensus immunoscore for the classification of colon cancer: a prognostic and accuracy study. *Lancet.* 2018;391(10135):2128-2139.

39. Crotty S. T follicular helper cell biology: a decade of discovery and diseases. *Immunity.* 2019;50(5):1132-1148.

40. Sage PT, Sharpe AH. T follicular regulatory cells. *Immunol Rev.* 2016;271(1):246-259.

41. DiToro D, Basu R. Emerging complexity in CD4(+)T lineage programming and its implications in colorectal cancer. *Front Immunol.* 2021;12:694833.

42. Salama P, Phillips M, Grieu F, et al. Tumor-infiltrating FOXP3+ T regulatory cells show strong prognostic significance in colorectal cancer. *J Clin Oncol.* 2009;27(2):186-192.

43. Zhuo C, Li Z, Xu Y, et al. Higher FOXP3-TSDR demethylation rates in adjacent normal tissues in patients with colon cancer were associated with worse survival. *Mol Cancer.* 2014;13:153.

44. Saito T, Nishikawa H, Wada H, et al. Two FOXP3(+)CD4(+) T cell subpopulations distinctly control the prognosis of colorectal cancers. *Nat Med.* 2016;22(6):679-684.

45. Mantovani A, Marchesi F, Malesci A, Laghi L, Allavena P. Tumour-associated macrophages as treatment targets in oncology. *Nat Rev Clin Oncol.* 2017;14(7):399-416.

46. Ostuni R, Kratochvill F, Murray PJ, Natoli G. Macrophages and cancer: from mechanisms to therapeutic implications. *Trends Immunol.* 2015;36(4):229-239.

47. Yang C, Wei C, Wang S, et al. Elevated CD163(+)/CD68(+) ratio at tumor invasive front is closely associated with aggressive phenotype and poor prognosis in colorectal cancer. *Int J Biol Sci.* 2019;15(5):984-998.

48. Kang JC, Chen JS, Lee CH, Chang JJ, Shieh YS. Intratumoral macrophage counts correlate with tumor progression in colorectal cancer. *J Surg Oncol.* 2010;102(3):242-248.

49. Kruse J, vonBernstorff W, Evert K, et al. Macrophages promote tumour growth and liver metastasis in an orthotopic syngeneic mouse model of colon cancer. *Int J Colorectal Dis.* 2013;28(10):1337-1349.

50. Oosterling SJ, van derBij GJ, Meijer GA, et al. Macrophages direct tumour histology and clinical outcome in a colon cancer model. *J Pathol.* 2005;207(2):147-155.

51. Wei C, Yang C, Wang S, et al. Crosstalk between cancer cells and tumor associated macrophages is required for mesenchymal circulating tumor cell-mediated colorectal cancer metastasis. *Mol Cancer.* 2019;18(1):64.

52. Li J, Li L, Li Y, et al. Tumor-associated macrophage infiltration and prognosis in colorectal cancer: systematic review and meta-analysis. *Int J Colorectal Dis.* 2020;35(7):1203-1210.

53. Xue T, Yan K, Cai Y, et al. Prognostic significance of CD163+ tumor-associated macrophages in colorectal cancer. *World J Surg Oncol.* 2021;19(1):186.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.