Infiltrating regulatory T cells promote invasiveness of liver cancer cells via inducing epithelial-mesenchymal transition

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Background: Regulatory T (Treg) cells are a major component of the microenvironment of hepatocellular carcinoma (HCC) contributing to immunosuppression. The present study aimed to evaluate the effects of Treg cells on the invasion potential of HCC.

Methods: Infiltrating Treg cells were isolated from fresh HCC tissues by immunomagnetic bead separation and detected by flow cytometry. Circulating tumor cells (CTCs) were detected using the CellSearch platform. The cell migration and invasion potentials were evaluated by Transwell assays. The cell viability was tested by the cell counting kit-8 (CCK8) approach, and the apoptosis rates were determined by flow cytometry. The concentrations of active transforming growth factor-β1 (TGFβ1) were measured by enzyme-linked immunosorbent assay.

Results: Infiltrating Treg cells significantly correlated with the number of CTCs and vascular invasion (both P<0.05). Moreover, these cells could greatly promote HCC migration, invasion, and proliferation, and inhibit HCC apoptosis. Polymerase chain reaction and Western blot assays revealed that Treg cells significantly decreased the expression levels of epithelium-related molecules and increased the expression levels of mesenchyme-related molecules. Treg cells could activate Smad2/3 via secreting TGFβ1, and these effects could be impaired by knocking down the expression of TGFβ1 in Treg cells.

Conclusions: The involvement of infiltrating Treg cells in triggering the TGFβ1 signaling pathway and promoting the epithelial-mesenchymal transition (EMT) of cancer cells during tumor hematogenous dissemination is presumably responsible for increasing the invasiveness potential of HCC cells. Targeting Treg cells in microenvironments can be a promising therapeutic strategy to improve the prognosis for patients with HCC undergoing resection.

Keywords: Circulating tumor cells; epithelial-mesenchymal transition (EMT); hepatocellular carcinoma (HCC); regulatory T cells; transforming growth factor-β1 (TGFβ1)

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide, with recently escalating incidence and generally dismal outcomes (1,2). By far, surgical resection is still considered as the first option for HCC cure (3). However, postoperative survival remains unsatisfactory because of high recurrence and metastasis rates due to the high invasiveness potential of HCC cells (4-6). Therefore, a better understanding of HCC cell spread may provide novel insights into HCC management in the future. The transforming growth factor-β1 (TGFβ1)-induced TGFβ signaling pathway has been reported to play a vital role in HCC progression and spread (7). Active TGFβ1 could trigger epithelial–mesenchymal transition (EMT) in a Smad2/3-dependent manner, greatly promoting the invasiveness of tumor cells (8-10). Previous studies reported that regulatory T (Treg) cells were a critical resource of TGFβ1 and T-reg-derived TGFβ1 could also promote tumor invasion (11,12). However, whether infiltrating Treg was an important origin of TGFβ1 in HCC, and its role in promoting HCC progression, remained elusive.

Recent data have provided solid evidence that the generation of circulating tumor cells (CTCs) was an early event in tumor metastasis, and these CTCs were considered as the “seeds” of metastasis (13,14). Accumulating reports have proven the role of CTCs in HCC metastasis cascade (15). CTCs expressing an epithelial cell adhesion molecule (EpCAM) could be detected in about half of patients with HCC, and patients with high EpCAM-CTCs have been proved to encounter poor outcomes, including a high incidence of recurrence and tumor-related death (15,16). However, the mechanism underlying the release of these cells from tumor bulks is extremely complicated and needs to be further investigated. Recent data revealed that the acquisition of invasiveness by interaction with mesenchymal cells, including Tregs, was essential for tumor cells, suggesting a novel strategy for investigating HCC progression (17).

In light of the aforementioned considerations, the correlation between infiltrating Treg cells and CTCs and the effects of Treg cells on HCC cells were further investigated via in vitro experiments. The data demonstrated that in situ Treg cells could significantly promote the invasiveness potentials of HCC cells via TGFβ1-induced Smad2/3 phosphorylation. Therefore, the findings indicated that infiltrating Treg cells served as a critical regulator of HCC invasion, and future therapeutic approaches targeting Treg cells might pave the novel way for curing HCC.

Methods

Cell lines and cell culture

The Huh7 cell line was purchased from the Cell Bank, Institute of Biochemistry and Cell Biology, China Academy of Science (Shanghai, China). The MHCC-97H cell line was a gift from the Liver Cancer Institute of Zhongshan Hospital, Fudan University (Shanghai, China). Both cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), supplemented with 100 IU/mL penicillin and 100 μg/mL streptomycin and incubated at 37 °C under a humidified atmosphere with 5% CO2. All cell culture reagents were obtained from Life Technology (Thermo Fisher Scientific, MA, USA).

Fresh clinical tissue specimens

A total of 40 fresh HCC tissues were collected at Liver Institute of Zhongshan Hospital, Fudan University (Shanghai, China). No previous local or systemic treatment had been conducted for these patients before treatment. Surgical specimens were obtained from all patients at the time of resection. All samples were received in the laboratory within 2 h, immediately mechanically disaggregated and digested with type 4 collagenase (Thermo Fisher Scientific), and then resuspended in DMEM. Single-cell suspensions were obtained by filtration through a 40-μm filter. Red blood cells were lysed with ACK buffer (Thermo Fisher Scientific). The viable cells were counted and analyzed using trypan blue. The approval for the use of human participants was obtained from the research ethics committee of Zhongshan Hospital, and informed consent was obtained from each individual.

Infiltrating lymphocyte isolation and detection

Human lymphocytes were isolated from fresh tissues by centrifugation through a discontinuous Percoll (GE Healthcare, Piscataway, New Jersey, USA) gradient with densities of 1.06 and 1.08 g/mL according to a previous study (18). Isolated lymphocytes were harvested for further experiments. Treg cells were detected by flow cytometry using a human CD4+CD25+FoxP3+ Treg detection kit (eBioscience, CA, USA) according to the manufacturer's protocols. Briefly, 20μL of anti-CD4-fluorescein isothiocyanate (FITC)/anti-CD25-APC (allophycocyanin) and the relevant isotype control antibody (IgG1K-
FITC and IgG1K-APC, respectively) were added into two separate flow cytometry tubes. Then, 100 μL of the suspension containing 10⁶ freshly isolated lymphocytes was added, followed by incubation at 4 °C for 30 min in the dark. Afterward, each tube was supplemented with 1 mL of permeabilization reagent and incubated at 4 °C for 60 min. After washing with phosphate-buffered saline (PBS), 100 μL of mouse serum was added and the mixture was incubated at room temperature (RT) in the dark for 15 min. Further, 20 μL of intracellular antibody anti-FoxP3-PE and isotype control IgG2a-K-PE were each added, and the resulting solution was incubated at RT in the dark for another 30 min. After resuspension in PBS, the sample was loaded for flow cytometry analysis. The measurement included proportions of CD4⁺CD25⁺FoxP3⁺ T cells (Tregs) in total lymphocytes and CD4⁺ T cells.

**Co-culture of Treg and tumor cells**

Treg cells were separated using a human CD4⁺CD25⁺ regulatory T cell isolation kit (Miltenyi, Germany) according to the manufacturer’s protocols. The purity of Treg cells was verified by flow cytometry. Afterward, 1×10⁵ Huh7 or 97H cells were seeded with 1×10⁵ Treg cells in 96-well plates with DMEM containing 10% FBS, using Huh7 or 97H cells without any lymphocytes as controls. The co-cultures were incubated for 72 h, and the tumor cell viability on each day was evaluated by CCK-8 assays (Dojindo Inc., Japan) according to the manufacturer’s protocols. All experiments were conducted in triplicate.

**Migration, invasion, cell cycle, and apoptosis assays**

For detecting migration and invasion, Transwell assays were used. Briefly, 1×10⁵ Huh7 or 97H cells in 100 μL of serum-free DMEM were added to the well of Boyden chamber with an 8-μm-pore membrane (Corning, Inc., NY, USA). The bottom chamber contained 10% FBS in DMEM as a chemoattractant. Further, 1×10⁶ Treg cells were added to the upper chambers. For evaluating migration, the cells were cultured without MatriGel, while for evaluating invasion, the cells were cultured with MatriGel. The cells were allowed to invade for 24 h (migration evaluation) or 72 h (invasion evaluation); the cells that had not penetrated the filters were then removed by scrubbing with cotton swabs. The untransfected cells were similarly treated and included as a negative control. The chambers were fixed for 20 min at RT with 4% formaldehyde in PBS, stained with 0.1% crystal violet for 30 min, and rinsed in water. The cells that migrated to the bottom surface of the filter were considered to have invaded through the matrix and were counted under a light microscope. The assays were performed three times using triplicate wells.

The cell cycle assays were conducted with flow cytometry, briefly, 3×10⁵ Huh7 or 97H cells were seeded in 6-well plates with 3×10⁶ Treg cells and co-cultured for 48 h. Then, the tumor cells were harvested and washed with PBS three times and suspended in cold PBS. The cells were stained with propidium iodide (PI)/RNase staining solution at 37 °C in the dark for 30 min. The cell cycle was performed in three replicates using flow cytometry (BD Biosciences, CA, USA).

Cell apoptosis was analyzed by flow cytometry using Annexin V–FITC. Apoptosis Detection Kits (BD Biosciences) were employed according to the manufacturer’s protocol. Briefly, 1×10⁵ Huh7 or 97H cells were seeded in 24-well plates with 1×10⁶ Treg cells; these cells were co-cultured for 48 h and then harvested and suspended in a binding buffer (1×). The cells were similarly treated without Treg cells as a negative control. An aliquot of 100 μL was incubated with 5 μL of Annexin V–FITC and 5 μL of PI for 15 min in the dark, and 400 μL of binding buffer (1×) was added to each sample. The stained cells were analyzed by flow cytometry within 1 h. All in vitro experiments were conducted in triplicate. Treg cells separated from HCC tissues were used for a single function assay in the present study.

**Detection of CTCs**

CTCs were detected using the CellSearch system, as previously described (16). In brief, the semiautomated CellSearch platform (Janssen Diagnostics, NJ, USA) enriched the sample for cells expressing the EpCAM with ferromagnetic beads. Afterward, fluorescently labeled monoclonal antibodies specific for leukocytes (CD45) and cytokeratins were used to distinguish epithelial cells from leukocytes. The identification and enumeration of CTCs were performed using the CellSpotter Analyzer, and the results were expressed as the number of cells per 7.5 mL of blood.

**RNA isolation and quantitative reverse transcription–polymerase chain reaction**

Total RNA was extracted from cell lines using the RNeasy
mini kit (Qiagen, Germany) according to the manufacturer's protocols. mRNA was reverse transcribed into cDNA using a QuantiTect Reverse Transcription Kit (Qiagen). The mRNA expression level in HCC cell lines was measured by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) using a LightCycle480 instrument (Roche Diagnostics, Germany). qRT-PCR was conducted using a LightCycle480 SYBR I Master Mix (Roche Diagnostics) according to the manufacturer's protocols. qRT-PCR was performed with an initial denaturation at 95 °C for 5 min followed by 40 cycles of denaturation at 95 °C for 15 s, annealing, and extension at 60 °C for 30 s. GAPDH was used as an internal control. The relative mRNA expression levels were calculated based on the Ct values and normalized using GAPDH expression, according to the equation: 2^{-\Delta \Delta Ct} \ [\Delta Ct = Ct (Target) – Ct (GAPDH)]. All experiments were performed in triplicate.

Cell transfection
The expression of KNPN3 was knocked down using a retroviral vector pMSCV. The retrovirus was produced by 293T packaging cells. The pLKO.1-shRNAs targeting TGFβ oligonucleotide were TGCTGTTGC-GAAGACATAGAAATGAGAAGCCACAGATGTATTCATTTCTATGTCTTCGCATTTGCCTACTGCCTCGGA. The cells were infected with these viruses or control virus and then selected against puromycin for 3 days before being split for further assays. The reporter plasmid and luciferase promoter were purchased from Promega (WI, USA). The results of the validation of TGFβ silencing are displayed in Figure S1.

Enzyme-linked immunosorbent assay
The concentration of TGFβ1 in the conditioned supernatant from the co-culture experiments and in the peripheral blood of patients or healthy individuals was determined using a sandwich enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's protocols (R&D systems, Minneapolis, USA).

Western blot analysis
Total protein of Huh7 or 97H cells was extracted in lysis buffer for 30 min on ice. Equal amounts of proteins were separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred to polyvinylidene difluoride membranes (Millipore, MA, USA) using a mini trans-blot apparatus (Bio-Rad, CA, USA). The membranes were blocked with Tris-buffered saline with Tween (TBST) containing 5% nonfat dry milk for 1 h and incubated with primary antibodies, E-cadherin (Cell Signaling Technology CST, Boston, USA, 1:1,000), Vimentin (CST, 1:1,000), Twist (CST, 1:1,000), α-SMA (CST, 1:1,000), Smad2/3 (CST, 1:500), pSmad2/3 (CST, 1:500), and β-Actin (CST, 1:1,000), overnight at 4 °C. The membranes were then washed three times with TBST and incubated with horseradish peroxidase–conjugated immunoglobulin G (Chemicon, MA, USA) at a 1:5,000 dilution for 1 h at RT. The blots were developed using an enhanced chemiluminescence kit (Pierce, WA, USA). Each experiment was repeated three times.

Statistical analysis
Statistical analyses were performed using SPSS 21.0 software (IBM, USA). The experimental values for continuous variables were expressed as the mean ± standard error of the mean. The chi-square test, Fisher’s exact probability tests, and the Student’s t-test were used as appropriate to evaluate the significance of differences in data between the groups. If variances within the groups were not homogeneous, the nonparametric Mann-Whitney test or the Wilcoxon signed-rank test was used. A P value less than 0.05 was considered statistically significant.

Results
Infiltrating Treg cells positively correlated with HCC invasiveness
To investigate the effect of infiltrating Tregs on HCC invasiveness, the correlation between Treg cells and CTCs was evaluated in patients with HCC. First, the purity and viability of the separated Treg cells was determined by puromycin for 3 days before being split for further assays. The reporter plasmid and luciferase promoter were purchased from Promega (WI, USA). The results of the validation of TGFβ silencing are displayed in Figure S1.
Figure 1 Infiltrating Treg cells positively correlated with circulating tumor cells and invasiveness phenotype of HCC. (A) Assessment of the purity (left) and apoptosis rate (right) of Treg cells using FACS. (B) Treg cell contents significantly correlated with the numbers of CTCs (P<0.001). (C) Treg cell contents significantly increased in patients with high CTC loads (CTC \( \geq 2 \) per 7.5 mL) (P<0.001), and patients with vascular invasion exhibited higher Treg cell contents (P=0.038).
infiltrating Treg cell contents were also compared between patients with or without vascular invasion. Similarly, patients with vascular invasion exhibited higher Treg cell contents (P=0.038, Figure 1C). Collectively, these findings indicated that infiltrating Treg cells positively correlated with HCC invasiveness.

**Effects of infiltrating Treg cells on HCC cell motility, proliferation, cell cycle, and apoptosis in vitro**

To further explore the effects of Treg cells on HCC cells, infiltrating Treg cells were isolated via magnetic-based approaches and then co-cultured with HCC cell lines. Transwell assays showed that the migration and invasion capacities were significantly promoted when co-cultured with Treg cells in both Huh7 and 97H cells (all P<0.050, Figure 2A). The effects of Treg cells on proliferation were also evaluated. Both Huh7 and 97H cells proliferated faster when co-cultured with Treg cells according to CCK-8 assays (Figure 2B). Whether Treg cells could promote cell cycle transition was further explored considering their proliferation-promoting ability. Based on the flow cytometry results, the cells co-cultured with Treg cells showed a higher proportion of S phase, indicating higher proliferation rates (Figure 2C). Finally, the effects of Treg cells on apoptosis were investigated, and the results showed that cell apoptosis was significantly inhibited when co-cultured with Treg cells (Figure 2D). Taken together, the results demonstrated that infiltrating Treg cells could promote cell motility and proliferation and inhibit cell apoptosis, inevitably resulting in HCC progression.

**Infiltrating Treg cells induced EMT in HCC cells**

EMT was considered as a key contributor for cell invasiveness. Thus, whether infiltrating Treg cells could induce EMT was investigated. When co-cultured with infiltrating Tregs, HCC cells showed significantly lower expression levels of E-cadherin and claudin1 mRNA, while the mRNA expression levels of mesenchymal biomarkers, including N-cadherin, Vimentin, and fibronectin increased (Figure 3A). Moreover, two critical molecules involved in EMT, Twist and Snail, also showed increased mRNA expression levels after Treg stimulation (Figure 3A). To validate these findings, Western blot (WB) assays were conducted, and the results showed that the expression level of E-cadherin decreased while that of Vimentin increased at the protein level when co-cultured with Treg cells (Figure 3B). Importantly, the expression level of Twist also increased by Treg stimulation according to the WB assay (Figure 3C).

**Infiltrating Treg cells induced EMT via secreting TGFβ1**

TGFβ1-induced Smad2/3 activation was reported to a major cause for EMT, while Treg cells were conventionally considered as an important resource of TGFβ1. Hence, it was speculated that Treg cells might trigger EMT via secreting TGFβ1. To confirm this, the TGFβ1 mRNA expression levels of Treg cells were detected in the peripheral blood (either healthy donors or patients with HCC) and intratumoral environment (infiltrating Treg cells). The infiltrating Treg cells exhibited the highest expression levels of TGFβ1 mRNA compared with Treg cells from the peripheral blood of both healthy donors (n=10) and patients with HCC (n=10) (Figure 4A). Meanwhile, the expression levels of Foxp3 mRNA showed no difference among the three groups (Figure 4A). Further, ELISA revealed that the supernatant of infiltrating Treg cells contained significantly higher concentrations of TGFβ1 compared with that of Treg cells from the peripheral blood of both healthy donors (n=10) and patients with HCC (n=20), while intratumoral TGFβ1 concentrations showed no significant difference compared with those in the supernatant of infiltrating Treg cells (Figure 4B). These findings indicated that the infiltrating Treg cells exhibited impressive TGFβ1 secretion ability and might serve as a major resource of TGFβ1 within the tumor microenvironment.

To validate the role of Treg cells in activating Smad2/3, WB assays were performed. Treg cells could greatly increase the phosphorylation levels of Smad2/3 and promote the expression of α-SMA, confirming the effect of Treg cells on Smad2/3 activation (Figure 5). Next, to verify the role of TGFβ1 in Treg-mediated Smad2/3 activation and EMT, the expression of TGFβ1 was knocked down via siRNA on isolated infiltrating Treg cells, which were then co-cultured with HCC cells. RT-PCR showed that Treg cells lost their function to induce EMT (Figure 5A). Meanwhile, WB assays indicated that TGFβ1-KD impaired the effect of Treg cells on Smad2/3 activation (Figure 5). Collectively, these data demonstrated that infiltrating Treg cells promoted cell invasiveness by secreting TGFβ1.

**Discussion**

Previous studies revealed that Treg cells significantly
**Figure 2** In vitro effects of infiltrating T reg cells on HCC cells. 
(A) Migration and invasion potentials of HCC cells were significantly promoted when co-cultured with T reg cells in both Huh7 and 97H cells (Crystal Violet Staining, Magnification, ×100. P<0.050). 
(B) Effects of infiltrating Tregs on the proliferation potentials of HCC cells detected using CCK8 assays. 
(C) Effects of infiltrating T reg cells on the cell cycle of HCC cells detected using FACS. Tregs showed a higher proportion of S phase. 
(D) Cell apoptosis was significantly inhibited when co-cultured with Treg cells.
Figure 3 Infiltrating Treg cells triggered EMT in HCC cells. (A) mRNA expression of EMT-related molecules evaluated using RT-PCR assays. The expression levels of mesenchymal biomarkers, including N-cadherin, Vimentin, and fibronectin increased (*P<0.05 vs. NC). Protein expression levels of E-cadherin and Vimentin (B) and Twist (C) in Huh7 and MHCC97H cells evaluated using WB assays. The expression levels of E-cadherin decreased while the expression levels of Vimentin and Twist increased at the protein level when co-cultured with Treg cells.

Figure 4 Infiltrating Treg cells secreted high levels of TGFβ1 and had high mRNA expression. Active TGFβ1 concentrations were detected by ELISA. mRNA expression levels of TGFβ1 and FoxP3 were detected by RT-PCR. Treg cells were obtained from the intratumoral environment and the peripheral blood of patients with HCC, and Treg cells from the peripheral blood of healthy donors were selected as control. (A) Treg cells exhibited the highest TGFβ1 mRNA expression levels compared with Tregs from the peripheral blood of healthy donors. Foxp3 mRNA expression levels showed no difference among the three groups. (B) Supernatant of infiltrating Treg cells contained significantly higher concentrations of TGFβ1 compared with those of Treg cells from the peripheral blood of healthy donors while no significant difference compared with intratumoral TGFβ1 (*P<0.01 vs. peripheral blood).
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EMT to promote tumor progression, and targeting T reg
cells in HCC might be a promising therapeutic approach
for preventing HCC progression.

Invasiveness is a major characteristic of HCC that
contributes to the high incidence of metastasis or recurrence
after treatment (24,25). The acquisition of invasion potential
is a complex process, and EMT is currently considered as
the critical step for this biological transformation (26-28).
The infiltrating T reg cells induced a mesenchyme-like,
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The activation of Smad2/3 is a hallmark for invasiveness
and HCC progression, while reversing this abnormal
activation is considered a promising therapeutic approach
for HCC. Previous studies demonstrated that TGFβ1
was a major stimulator for activating Smad2/3 signaling,
including HCC (29). The present study found that
infiltrating T reg cells could activate Smad2/3 via secreting
TGFβ1, thus greatly triggering the TGFβ1 signaling
pathway. Importantly, T reg cells were identified as the
major source of intratumoral TGFβ1. The results clarified
the mechanism by which infiltrating T reg cells positively
controlled EMT and suggested a novel, promising target
for inhibiting TGFβ1 signaling to improve the prognosis of
patients with HCC.

Hematogenous spread is an important cause of
recurrence and metastasis in HCC, and CTCs in the
bloodstream plays an important role in HCC metastasis (30).
Meanwhile, the release of tumor cells into circulation is a
multiple-step process, and T reg acts as one of the major
regulators involved in this process, which could affect the
viability of HCC cells. A positive correlation was observed
between the infiltrating T reg cells and the number of peripheral CTCs in patients with HCC, indicating that
T reg cells might be a critical switch for CTC release.
Considering the role of T reg cells in mediating TGFβ1
signaling activation, intratumoral T regs could be a
promising therapeutic target to hinder CTC release and
directly prevent HCC metastasis and recurrence. However,
the clinical data of the correlation between T reg cells and
metastasis/recurrence was not provided in the present
study due to the lack of follow-up data. A prospective
cohort study is ongoing in the center to address this issue.
Although a previous study indicated that TGFβ1 might act
as a suppressor in HCC (31), several studies revealed the
pro-tumor effects of TGFβ1 in HCC development (32,33).
Meanwhile, considering its critical role in promoting cell
invasiveness (34), TGFβ1 might serve as a key regulator
of invasion in HCC, which was consistent with previous
findings. The present study also showed that TGFβ1
secreted from T reg cells could significantly promote the
invasiveness of HCC cells.

This study was novel in demonstrating the significance
of infiltrating T reg cells in mediating HCC motility and
progression. The data indicated that T reg cells could serve
as a novel target for preventing CTC release and HCC
metastasis. The involvement of infiltrating T reg cells in
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Figure 5 Western blot assays were conducted to detect the
expression level of α-SMA and phosphorylation level of Smad2/3;
β-actin was selected as a control. The TGFβ1 signaling pathway
was activated by Treg-derived TGFβ1. T reg cells could greatly
increase the phosphorylation levels of Smad2/3 and promote the
expression of α-SMA.

correlated with the prognosis of HCC (19,20). In addition,
they correlated with the invasiveness potential of HCC
(21-23). The present study found that infiltrating T reg cells
were positively correlated with the number of CTCs and
vascular invasion. In vitro experiments revealed that T reg
cells could promote the invasiveness potential of HCC
cells through secreting TGFβ1, thus triggering EMT. Moreover,
T reg cells also significantly promoted cell proliferation and
hindered cell apoptosis. The present data were consistent
with previous findings and could serve as a translational
basis for future anti-T reg therapeutic strategy.

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expression of α-SMA.
EMT of cancer cells during tumor hematogenous dissemination is presumably responsible for increasing the invasiveness potentials of HCC cells. Furthermore, targeting infiltrating Treg cells in the microenvironments of patients with HCC might be a promising therapeutic strategy to reduce the recurrence and metastasis for patients with HCC undergoing resection.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethics Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was approved by the ethics committee of Zhongshan Hospital (Reference number Y2014–028), and written informed consent was obtained from the patients for the publication of this manuscript and any accompanying images.

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Figure S1 The validation of TGFβ knockdown was confirmed by Western-blot analysis.