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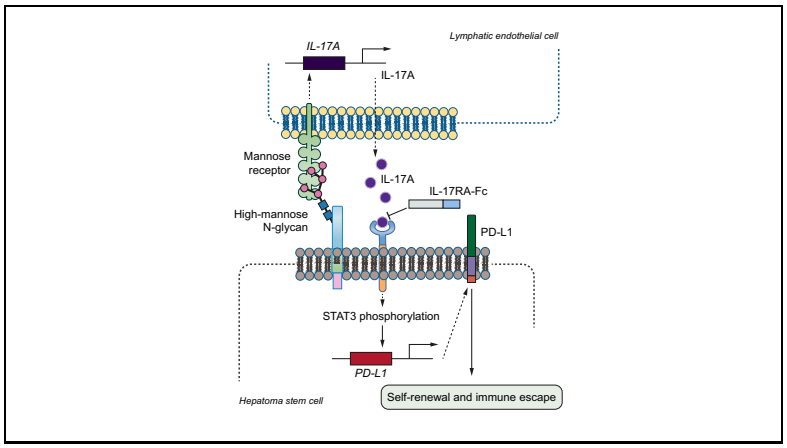
IL-17A secreted from lymphatic endothelial cells promotes tumorigenesis by upregulation of PD-L1 in hepatoma stem cells

Graphical abstract



Highlights

- Hepatoma stem cells preferentially interact with lymphatic endothelial cells.
- Interaction of hepatoma stem cells with lymphatic endothelial cells upregulates IL-17A.
- IL-17A promotes hepatoma stem cell self-renewal and immune escape.
- IL-17A is highly expressed in hepatoma.

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Lay summary

The microenvironment is crucial for the self-renewal and development of hepatoma stem cells, which lead to the development of liver cancer. Lymphatic endothelial cells are an important component of this niche microenvironment, helping hepatoma stem cells to self-renew and escape immune attack, by upregulating IL-17A signaling. Thus, targeting IL-17A signaling is a potential strategy for the treatment of hepatoma.

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IL-17A secreted from lymphatic endothelial cells promotes tumorigenesis by upregulation of PD-L1 in hepatoma stem cells

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Background & Aims: The microenvironment regulates hepatoma stem cell behavior. However, the contributions of lymphatic endothelial cells to the hepatoma stem cell niche remain largely unknown; we aimed to analyze this contribution and elucidate the mechanisms behind it.

Methods: Associations between lymphatic endothelial cells and $CD133^+$ hepatoma stem cells were analyzed by immunofluorescence and adhesion assays; with the effects of their association on IL-17A expression examined using western blot, quantitative reverse transcription PCR and luciferase reporter assay. The effects of IL-17A on the self-renewal and tumorigenesis of hepatoma stem cells were examined using sphere and tumor formation assays. The role of IL-17A in immune escape by hepatoma stem cells was examined using flow cytometry. The expression of IL-17A in hepatoma tissues was examined using immunohistochemistry.

Results: $CD133^+$ hepatoma stem cells preferentially interact with lymphatic endothelial cells. The interaction between the mannose receptor and high-mannose type N-glycans mediates the interaction between $CD133^+$ hepatoma stem cells and lymphatic endothelial cells. This interaction activates cytokine IL-17A expression in lymphatic endothelial cells. IL-17A promotes the self-renewal of hepatoma stem cells. It also promotes their immune escape, partly through upregulation of PD-L1.

Conclusion: Interactions between lymphatic endothelial cells and hepatoma stem cells promote the self-renewal and immune escape of hepatoma stem cells, by activating IL-17A signaling. Thus, inhibiting IL-17A signaling may be a promising approach for hepatoma treatment.

Lay summary: The microenvironment is crucial for the self-renewal and development of hepatoma stem cells, which lead to the development of liver cancer. Lymphatic endothelial cells are an important component of this niche microenvironment, helping hepatoma stem cells to self-renew and escape immune attack, by upregulating IL-17A signaling. Thus, targeting IL-17A signaling is a potential strategy for the treatment of hepatoma.

Introduction

Hepatoma stem cells are thought to be responsible for the initial development, progression and therapeutic resistance of hepatomas.[1] For example, $CD133^+$ hepatoma cells highly express stemness genes, possess multi-lineage differentiation capabilities and are highly tumorigenic in immunocompromised mice.[2,3] Exploring the mechanisms behind the highly tumorigenic nature of hepatoma stem cells will contribute to the development of therapeutic approaches for hepatocellular carcinoma.

Cancer stem cells (CSCs) exist in a cellular niche comprised of numerous cell types.[4,5] For example, tumor-associated macrophages promote the survival and self-renewal of CSCs.[6] Lymphatic vessels are one of the major routes by which cancer cells disseminate.[7] Increasing evidence has shown that lymphatic endothelial cells regulate tumor cell behaviors. For example, lymphatic endothelial cells recruit CXCR4- or CCR7-expressing cancer cells by chemo-attraction.[8] In addition, lymphatic endothelial cells secrete factors to support tumor cell proliferation.[9] Thus, lymphatic endothelial cell serves as an important component of the tumor microenvironment. However, the mechanisms underlying the crosstalk between CSCs and lymphatic endothelial cells remain largely unknown.

Here, we examined the mechanism by which lymphatic endothelial cells promoted the self-renewal of hepatoma stem cells. We found that lymphatic endothelial cells create a CSC-niche through direct contact with hepatoma stem cells. This niche promotes the self-renewal and immune escape of hepatoma stem cells.

Material and methods

Isolation of $CD133^+$ and $CD133^-$ cells

$CD133^+$ cells were isolated from xenografts formed by hepatoma cell lines or human hepatoma tissues as previously

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described.[10] Human hepatoma tissues were obtained in accordance with protocols approved by the Ethics Committees of School of Basic medical of Fudan University. CD133$^+$ and CD133$^-$ cells were separated through magnetic cell sorting with a CD133 Cell Isolation Kit (Miltenyi Biotec).

Tumor-initiating capacity of tumor cells

To examine the tumor-initiating capacity of tumor cells, tumor cells were orthotopically transplanted into 7-week old immunodeficient mice in accordance with protocols approved by School of Basic medical of Fudan University Animal Care and Use Committee. Tumor formation was determined by histology.

Cell culture

CD133$^+$ cells were cultured in the DMEM/F12 media supplemented with B27 lacking vitamin A (Invitrogen), 2 μg/ml heparin (Sigma), 20 ng/ml EGF (Chemicon) and 10 ng/ml FGF-2 (Chemicon). CD133$^-$ tumor cells were plated in DMEM with 10% fetal bovine serum for at least 12 h to permit cell survival.

Dual-luciferase assay

Cells were co-transfected with pGL3-*IL-17A* or pGL3-*PD-L1* and pRL-SV40 plasmids as previously described.[11] Cells were lysed in a Passive Lysis Buffer (Promega) 48 h after transfection. Luciferase activities were measured using Dual-Luciferase® Reporter Assay System (Promega) with Turner luminometer and normalized to the *Renilla* luciferase activity for transfection efficiency. Data were represented as the mean from at least 3 independent experiments.

Statistical analysis

In general, significance was tested by paired or unpaired 2-tailed Student's *t* test or ANOVA test. *P* values <0.05 were considered statistically significant. Results are expressed as the mean ± SEM. The correlation between IL-17A expression and PD-L1 expression in hepatoma tissues was analyzed using Spearman's rank correlation test.

For further details regarding the materials and methods used, please refer to the CTAT table and supplementary information.

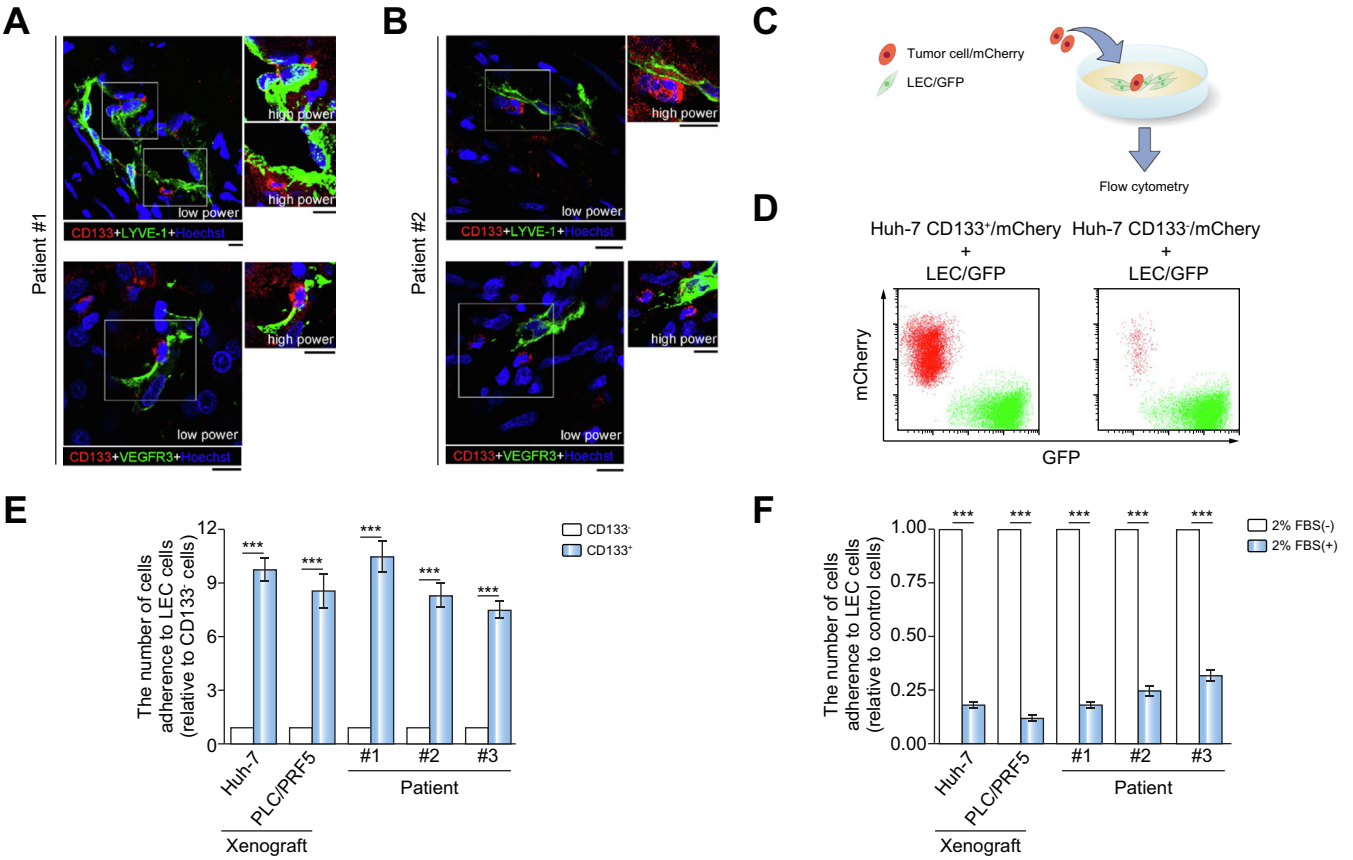


Fig. 1. Lymphatic endothelial cells interact with CD133$^+$ hepatoma cells. (A-B) Confocal images of liver tumor sections of patient #1 (A) and #2 (B) co-stained for cancer stem cell marker CD133 (red) and lymphatic endothelial cell marker LYVE-1 (upper panel) or VEGFR3 (lower panel) (green) showed juxtaposition of hepatoma stem cells with lymphatic endothelial cells. The high-power image of the framed regions in the left panels is shown in the right panels. Scale bar, 10 μM. (C) mCherry-labeled cells were co-cultured with GFP-labeled lymphatic endothelial cells. The number of adherent mCherry-labeled cells was quantified by flow cytometry based on Cherry expression and GFP expression. (D) Representative images of flow cytometry analysis of mCherry-labeled CD133$^+$ Huh-7 cells adherence to GFP-labeled lymphatic endothelial cells. (E) The ratios of mCherry-labeled CD133$^+$ or CD133$^-$ cells, isolated from Huh-7 and PLC/PRF/5 xenografts or HCC samples (patient #1-#3), that adhered to lymphatic endothelial cells, isolated from HCC tissues, were calculated from the flow cytometry quantitation. Results are expressed as mean ± SEM (n = 4). Data analyzed by 2-tailed *t* tests, $^{***}p$ <0.001. (F) The ratios of CD133$^+$ cells, isolated from xenografts or HCC samples pretreated with or without 2% FBS for 7 days, that adhered to lymphatic endothelial cells were calculated from the flow cytometry quantitation. Results are expressed as mean ± SEM (n = 4). Data analyzed by 2-tailed *t* tests, $^{***}p$ <0.001. HCC, hepatocellular carcinoma. (This figure appears in colour on the web.)

Results

Interaction between hepatoma stem cells and lymphatic endothelial cells

To examine the association between hepatoma stem cells and lymphatic endothelial cells, hepatoma tissues were analyzed by immunofluorescence with anti-CD133 (hepatoma stem cell marker) and anti-LYVE-1 or anti-VEGFR3 (lymphatic endothelial cell marker) antibodies. Juxtaposition of lymphatic endothelial cells with $CD133^+$ hepatoma cells was detected in hepatoma tissues (Fig. 1A-B). To examine whether hepatoma stem cells adhered to lymphatic endothelial cells, $CD133^+$ or $CD133^-$ cells were isolated from Huh-7 and PLC/PRF/5 xenograft, or HCC samples (patient #1-#3). Consistent with previous reports,[2,12] $CD133^+$ hepatoma cells showed characteristics consistent with CSCs, including sphere formation (Fig. S1A), expressing stem cell markers (Fig. S1B-F), multi-lineage differentiation capacity (Fig. S1G), and highly tumorigenic potential in immunocompromised mice (Fig. S1H-I). Human lymphatic endothelial cells were isolated from HCC samples by immunomagnetic purification as previously described.[13] Isolated lymphatic endothelial cells expressed lymphatic endothelial cell markers VEGFR3, LYVE-1 and podoplanin (Fig. S1J), and formed tube-like structures that were similar to lymphatic endothelial cells (Fig. S1K).

To examine the adherence of hepatoma stem cells to lymphatic endothelial cells, $CD133^+$ or $CD133^-$ cells isolated from Huh-7 and PLC/PRF/5 xenografts or HCC samples (patient #1-#3) labeled with mCherry (red fluorescent protein) were laid on top of lymphatic endothelial cells labeled with GFP (green fluorescent protein) (Fig. 1C). Compared to $CD133^-$ hepatoma cells, more $CD133^+$ hepatoma cells isolated from xenografts or HCC samples adhered to lymphatic endothelial cells (Fig. 1D-E). To examine the ability of hepatoma stem cells to adhere to

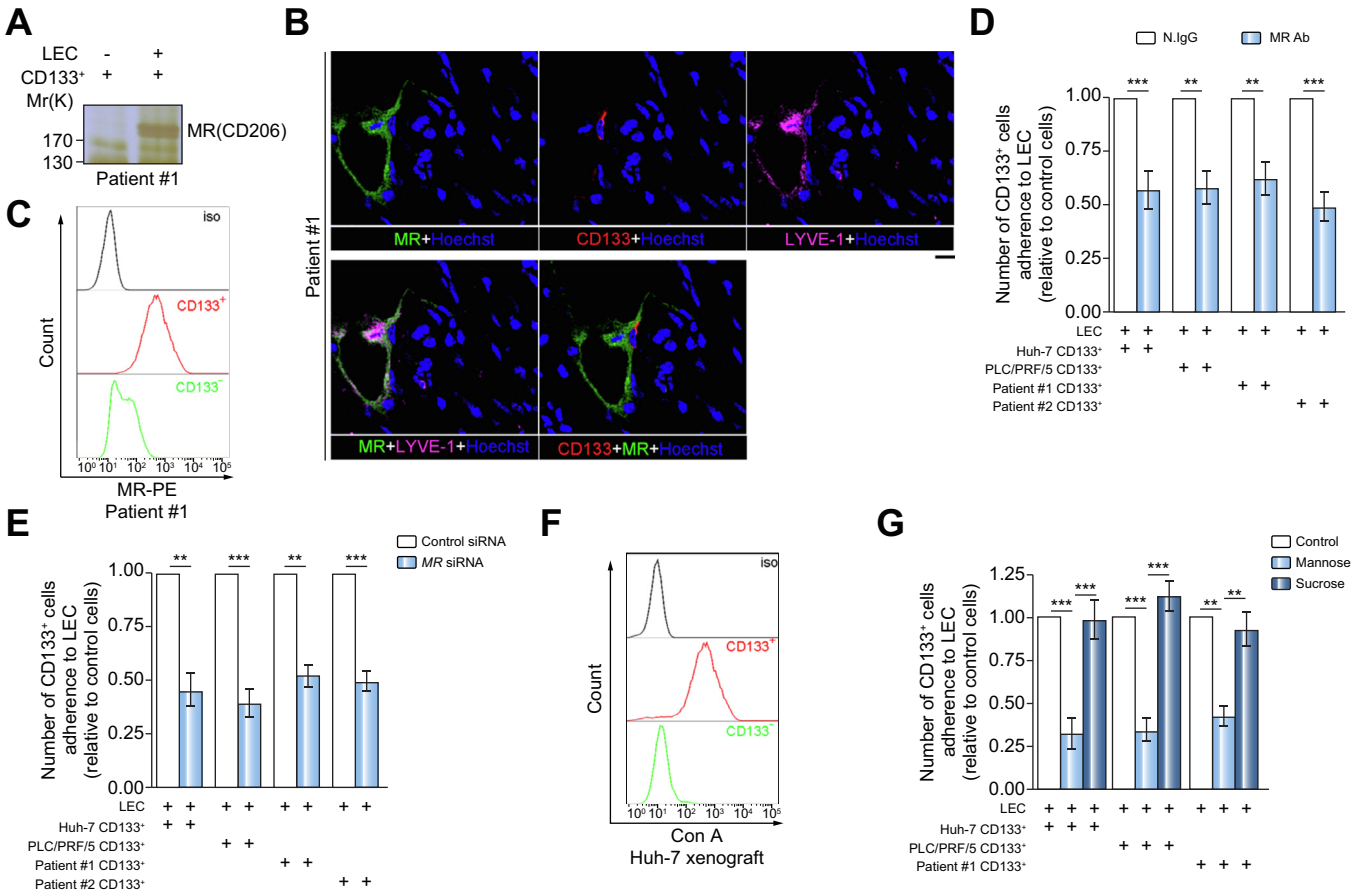


Fig. 2. Mannose receptor mediates the adherence of $CD133^+$ hepatoma cells to lymphatic endothelial cells. (A) Membrane lysis of biotinylated $CD133^+$ cells isolated from HCC sample (patient #1) were incubated with membrane lysis of lymphatic endothelial cells isolated from hepatoma tissues. The proteins that interacted with $CD133^+$ cells were purified by avidin agarose and then subjected to LC-MS/MS analysis. Peptide sequences of mannose receptor were identified by LC-MS/MS analysis. (B) Confocal images of the distribution of CD133 (red) and MR (green) and LYVE-1 (pink) in patient #1 HCC section. Scale bar, 10 μM. (C) Flow cytometry analysis of the binding of phycoerythrin (PE)-conjugated mannose receptor to $CD133^+$ cells or $CD133^-$ cells isolated from patient #1 HCC tissue. (D) The ratio of mCherry-labeled $CD133^+$ cells, isolated from Huh-7 or PLC/PRF/5 xenografts or HCC tissues (patients #1-#2), that adhered to GFP-labeled lymphatic endothelial cells pretreated with control IgG or anti-MR antibody were calculated from flow cytometry quantitation. Results are expressed as mean ± SEM (n = 4). Data analyzed by 2-tailed *t* tests, $^{**}p$ <0.01, $^{***}p$ <0.001. (E) The ratio of mCherry-labeled $CD133^+$ cells, isolated from Huh-7 and PLC/PRF/5 xenografts or HCC tissues (patient #1-#2), that adhered to GFP-labeled lymphatic endothelial cells expressing control siRNA or *MR* siRNA were calculated from flow cytometry quantitation. Results are expressed as mean ± SEM (n = 4). Data analyzed by 2-tailed *t* tests, $^{**}p$ <0.01, $^{***}p$ <0.001. (F) $CD133^+$ or $CD133^-$ cells isolated from Huh-7 xenografts were incubated with biotinylated Con A, followed by incubation with FITC-conjugated streptavidin. Analysis was performed using flow cytometry. (G) The ratios of mCherry-labeled $CD133^+$ hepatoma cells, isolated from Huh-7 or PLC/PRF/5 xenografts or patient #1 HCC sample pretreated with mannose or sucrose, that adhered to GFP-labeled lymphatic endothelial cells were calculated from flow cytometry quantitation. Results are expressed as mean ± SEM (n = 4). Data analyzed by 2-tailed *t* tests, $^{**}p$ <0.01, $^{***}p$ <0.001. HCC, hepatocellular carcinoma; LC-MS, liquid chromatography-mass spectrometry; MR, mannose receptor. (This figure appears in colour on the web.)

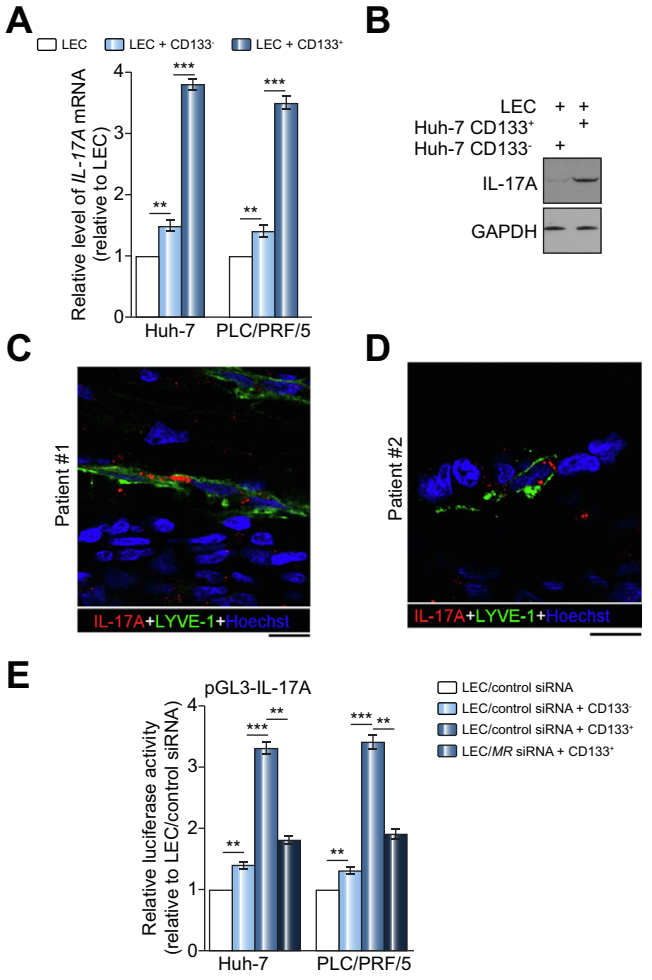


Fig. 3. The interaction between lymphatic endothelial cells and CD133⁺ hepatoma stem cells promotes IL-17A expression. (A) qRT-PCR analysis of *IL-17A* mRNA level in lymphatic endothelial cells co-cultured with CD133⁺ cells or CD133⁻ cells isolated from Huh-7 or PLC/PRF/5 xenografts. The ratio of *IL-17A* mRNA level to *GAPDH* mRNA level was expressed as mean ± SEM (n = 4). Data analyzed by 2-tailed *t* tests, $^{**}p$ <0.01, $^{***}p$ <0.001. (B) Western blot analysis of IL-17A in lymphatic endothelial cells co-cultured with CD133⁺ cells or CD133⁻ cells isolated from Huh-7 xenograft. (C-D) Confocal images of the distribution of IL-17A (red) and LYVE-1 (green) in the hepatoma sections of patient #1 (C) and #2 (D). Scale bar, 10 μM. (E) Lymphatic endothelial cells expressing control siRNA or mannose receptor siRNA transfected with pGL3-*IL-17A* and pRL-SV40 were co-cultured with CD133⁺ cells or CD133⁻ cells isolated from Huh-7 or PLC/PRF/5 xenografts. Luciferase activity was determined 48 h later, and was then standardized to that of lymphatic endothelial cells expressing control siRNA. Results are expressed as mean ± SEM (n = 5). Data analyzed by ANOVA tests, $^{**}p$ <0.01, $^{***}p$ <0.001. qRT-PCR, quantitative reverse transcription PCR. (This figure appears in colour on the web.)

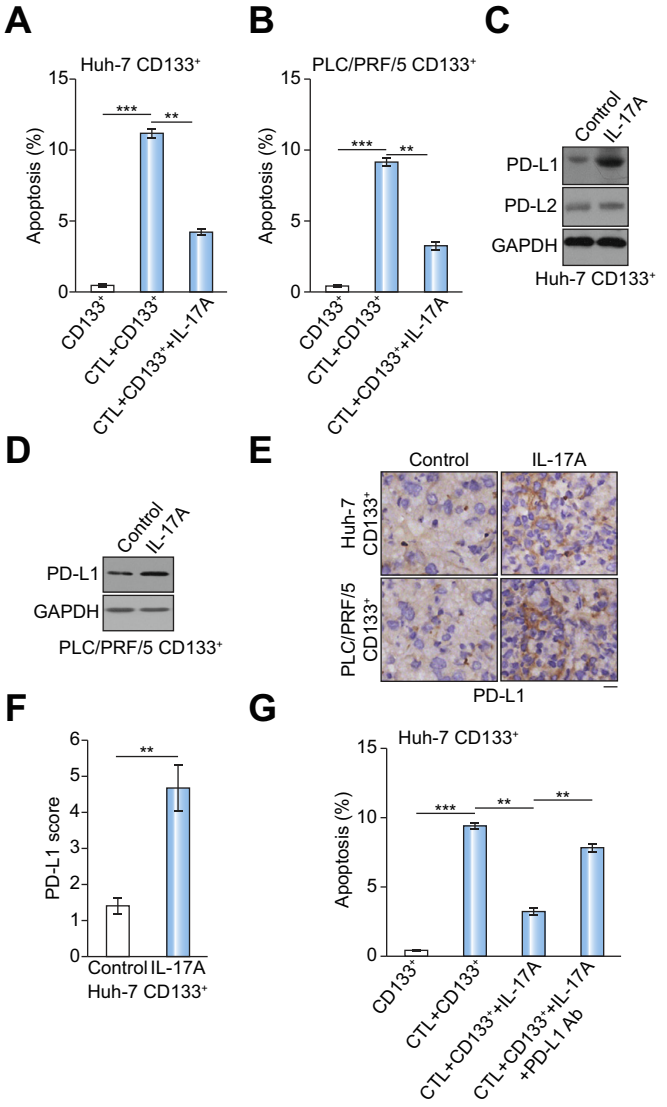


Fig. 4. IL-17A promotes the resistance of hepatoma stem cells to cytotoxic T cells through upregulation of PD-L1. (A-B) CD133⁺ cells isolated from Huh-7 xenograft (A) or PLC/PRF/5 xenograft (B) pretreated with or without IL-17A were co-cultured with activated CTLs for 4 h. The percentage of apoptotic CD133⁺ cells was determined by flow cytometry. Results are expressed as mean ± SEM (n = 4). Data analyzed by 2-tailed *t* tests, $^{**}p$ <0.01, $^{***}p$ <0.001. (C-D) Western blot analysis of PD-L1 expression in CD133⁺ cells isolated from Huh-7 xenograft (C) or PLC/PRF/5 xenograft (D) treated with IL-17A for 24 h. (E-F) Immunohistochemical staining for PD-L1 in xenograft formed by CD133⁺ cells isolated from Huh-7 xenograft (upper panel) or PLC/PRF/5 xenograft (lower panel) treated with or without IL-17A. (E) A representative image was shown. Scale bar represents 10 μM. (F) The scores for quantitative staining of PD-L1 in the tissue sections were expressed as mean ± SEM (n = 5) Data analyzed by 2-tailed *t* tests, $^{***}p$ <0.001. (G) CD133⁺ cells isolated from Huh-7 xenograft pretreated with or without IL-17A were co-cultured with CTLs for 4 h in the presence of anti-PD-L1 antibody. The percentage of apoptotic CD133⁺ cells was determined by flow cytometry. Results are expressed as mean ± SEM (n = 4). Data analyzed by ANOVA tests, $^{**}p$ <0.01, $^{***}p$ <0.001). CTLs, cytotoxic T lymphocytes. (This figure appears in colour on the web.)

lymphatic endothelial cells during differentiation, *in vitro* differentiation of CD133⁺ cells was induced by 2% serum. Compared to differentiated cells induced by 2% serum, more CD133⁺ hepatoma cells isolated from xenografts or HCC samples adhered to lymphatic endothelial cells (Fig. 1F). Thus, CD133⁺ hepatoma stem cells preferentially interact with lymphatic endothelial cells.

Mannose receptor mediates the interaction between CD133⁺ hepatoma cells and lymphatic endothelial cells

To examine the mechanism of the interaction between CD133⁺ hepatoma cells and lymphatic endothelial cells, plasma membrane proteins of biotinylated CD133⁺ cells isolated from patient #1 sample were incubated with plasma membrane extraction of lymphatic endothelial cells. The proteins which interacted with CD133⁺ cells were purified by NeutrAvidin agarose and were then subjected to LC-MS/MS analysis. The peptides sequences matched the mannose receptor (CD206) (Fig. 2A). Immunofluorescence analysis showed that the

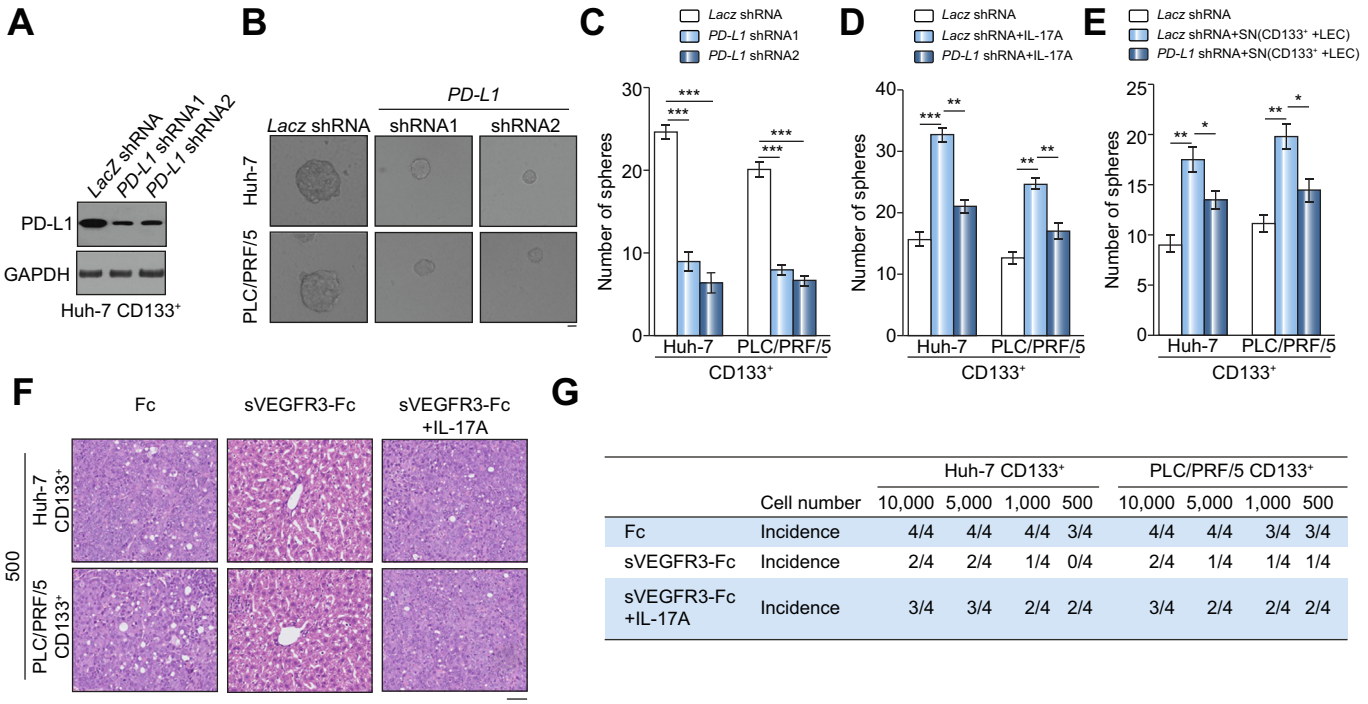
mannose receptor was expressed on lymphatic endothelial cells and co-localized with CD133$^+$ cells in human hepatoma tissues (Fig. 2B). By flow cytometry analysis, the binding of PE-tagged mannose receptor protein to CD133$^+$ cells isolated from HCC samples (patient #1-#2) was higher than to CD133$^-$ cells (Fig. 2C; Fig. S2A). Treatment with anti-mannose receptor antibody reduced the adherence of CD133$^+$ cells isolated from xenograft (Huh-7 or PLC/PRF/5) or HCC samples (patient #1-#2) to lymphatic endothelial cells (Fig. 2D). Downregulation of mannose receptor by siRNA in lymphatic endothelial cells (Fig. S2B), inhibited the adherence of CD133$^+$ cells isolated from xenograft (Huh-7 or PLC/PRF/5) or HCC samples (patient #1 and #2) to lymphatic endothelial cells (Fig. 2E).

Mannose receptor, a member of C-type lectins, binds to terminal high-mannose-type oligosaccharide.[14,15] N-glycans are mainly classified into 3 types: high-mannose type, hybrid type and complex type (Fig. S2C).[16,17] To examine the structural characteristics of N-glycan in CD133$^+$ cells, flow cytometry analysis was performed using FITC-conjugated PHA-L which interacts with the Galβ1 → 4GlcNAcβ1 → 6Man branch, or FITC-conjugated ConA which preferentially interacts with high-mannose N-glycans. Compared to CD133$^-$ cells, CD133$^+$ hepatoma cells isolated from Huh-7 xenograft or HCC sample (patient #2) were found to have high levels of high-mannose type N-glycans (Fig. 2F; Fig. S2D-F). Compared to differentiated cells induced by 2% FBS, CD133$^+$ hepatoma cells isolated from Huh-7 xenograft or HCC patient #1 sample were found to have high levels of high-mannose type N-glycans (Fig. S2G-J). Thus, the N-glycans on CD133$^+$ hepatoma stem cells might be predominantly high-mannose type N-glycans.

The addition of mannose, but not of sucrose, obviously inhibited the adherence of CD133$^+$ cells isolated from xenograft (Huh-7 or PLC/PRF/5) or patient #1 HCC sample to lymphatic endothelial cells (Fig. 2G). Consistent with this, treatment with Con A lectin inhibited the adherence of CD133$^+$ cells isolated from xenografts (Huh-7 or PLC/PRF/5) to lymphatic endothelial cells (Fig. S2K). Thus, the interaction between the mannose receptor and high-mannose type N-glycan promotes the interaction between CD133$^+$ hepatoma cells and lymphatic endothelial cells.

The co-culture of CD133$^+$ hepatoma cells with lymphatic endothelial cells promotes IL-17A expression

The interaction between the mannose receptor and its ligand promotes IL-17A expression.[18] Mannose treatment increased the expression of IL-17A in lymphatic endothelial cells, which could be blocked by mannose receptor siRNA (Fig. S3A), indicating that the binding of mannose to the mannose receptor promotes IL-17A expression in lymphatic endothelial cells. Compared to CD133$^-$ cells, the co-culture of CD133$^+$ hepatoma cells, isolated from xenografts, with lymphatic endothelial cells significantly increased *IL-17A* mRNA level and protein level (Fig. 3A-B) and



		Huh-7 CD133$^+$				PLC/PRF/5 CD133$^+$			
	Cell number	10,000	5,000	1,000	500	10,000	5,000	1,000	500
Fc	Incidence	4/4	4/4	4/4	3/4	4/4	4/4	3/4	3/4
sVEGFR3-Fc	Incidence	2/4	2/4	1/4	0/4	2/4	1/4	1/4	1/4
sVEGFR3-Fc +IL-17A	Incidence	3/4	3/4	2/4	2/4	3/4	2/4	2/4	2/4

Fig. 5. IL-17A promoted self-renewal of hepatoma stem cells through upregulation of PD-L1. (A) Western blot analysis of PD-L1 expression in CD133$^+$ cells isolated from Huh-7 xenograft expressing control shRNA or *PD-L1* shRNA. GAPDH expression served as a loading control. (B-C) Single cell sphere formation assay of CD133$^+$ cells isolated from Huh-7 xenograft expressing LacZ shRNA or *PD-L1* shRNA. (B) Representative images of sphere are shown. Scale bar, 10 μM. (C) Results are expressed as mean ± SEM (n = 5). Data analyzed by 2-tailed *t* tests, ***p <0.001. (D) Single cell sphere formation assay of CD133$^+$ cells isolated from Huh-7 or PLC/PRF/5 xenografts expressing LacZ shRNA or *PD-L1* shRNA treated with IL-17A. Results are expressed as mean ± SEM (n = 5). Data analyzed by 2-tailed *t* tests, **p <0.01, ***p <0.001. (E) Single cell sphere formation assay of CD133$^+$ cells isolated from HCC samples (#1 and #2) expressing LacZ shRNA or *PD-L1* shRNA treated with the supernatant from the co-culture of CD133$^+$ cells and lymphatic endothelial cells. Results are expressed as mean ± SEM (n = 5). Data analyzed by 2-tailed *t* tests, *p <0.05, **p <0.01). (F-G) The tumor-initiating capacity of CD133$^+$ cells isolated from Huh-7 or PLC/PRF/5 xenografts treated with sVEGFR3-Fc and/or IL-17A protein. Mice were sacrificed when they were moribund or 90 days after implantation. Tumor formation was determined by histology. (F) H&E staining of mouse liver shows tumors formation by 500 CD133$^+$ cells isolated from Huh-7 or PLC/PRF/5 xenograft treated with sVEGFR3-Fc and/or IL-17A-Fc protein. Scale bar, 25 μM. (G) The table displays number of mice developing tumors. HCC, hepatocellular carcinoma; SN, supernatant. (This figure appears in colour on the web.)

promoted IL-17A secretion (Fig. S3B). Immunofluorescence staining showed the positive expression of IL-17A in lymphatic endothelial cells in hepatoma tissues (Fig. 3C-D).

To examine the mechanism of co-culture of CD133$^+$ hepatoma cells with lymphatic endothelial cells promoting IL-17A expression, lymphatic endothelial cells expressing reporter vector pGL3-*IL-17A*-promoter were co-cultured with CD133$^+$ cells or CD133$^-$ cells isolated from xenograft (Huh-7 or PLC/PRF/5). Compared to CD133$^-$ cells, the co-culture of CD133$^+$ cells with lymphatic endothelial cells increased the activity of the *IL-17A* promoter (Fig. 3E), which could be reduced by downregulation of the mannose receptor in lymphatic endothelial cells (Fig. 3E). Consistent with this, treatment with Con A lectin reduced the activation of the *IL-17A* promoter by co-culture of CD133$^+$ cells with lymphatic endothelial cells (Fig. S3C). Thus, the interaction between lymphatic endothelial cells and hepatoma stem cells promotes *IL-17A* transcription by activating mannose receptor signaling.

IL-17A was derived mainly from CD4$^+$ T cells and γδT cells in cancer.[19–21] Using a quantitative reverse transcription PCR (qRT-PCR) assay, the expression of IL-17A in lymphatic endothelial cells was lower than in CD4$^+$ T cells, but higher than in natural killer cells or vascular endothelial cells (Fig. S3D). However, the mannose receptor was highly expressed in lymphatic endothelial cells as compared to CD4$^+$ T cells or vascular endothelial cells or natural killer cells (Fig. S3E). Together, the co-culture of CD133$^+$ hepatoma stem cells with lymphatic endothelial cells promotes IL-17A expression.

IL-17A promotes the immune escape of CD133$^+$ hepatoma cells through upregulation of PD-L1

CSCs are more resistant to immunological control than non-CSCs.[22,23] CD8$^+$ T cells (cytotoxic T cells) play a central role in immunity to cancer.[24] To examine the effect of IL-17A on the immune escape of hepatoma stem cells, CD133$^+$ cells isolated from Huh-7 or PLC/PRF/5 xenografts were pretreated with IL-17A and then co-cultured with activated CD8$^+$ T cells. IL-17A reduced the percentage of apoptotic CD133$^+$ cells isolated from xenografts induced by CD8$^+$ T cells (Fig. 4A-B). Supernatant from the co-culture of CD133$^+$ cells, isolated from Huh-7 xenografts, with lymphatic endothelial cells reduced the percentage of apoptotic CD133$^+$ cells induced by CD8$^+$ T cells. This process was inhibited by downregulation of IL-17A or mannose receptor in lymphatic endothelial cells (Fig. S4A). Thus, IL-17A from lymphatic endothelial cells promotes resistance of CD133$^+$ hepatoma stem cells to CD8$^+$ T cells.

The interaction of PD-L1/2 on the tumor cells with PD-1 on CD8$^+$ T cells reduces CD8$^+$ T cells function.[25,26] IL-17A induced PD-L1 expression in CD133$^+$ cells without significantly changing PD-L2 expression (Fig. 4C-D). Immunohistochemical staining showed that the expression of PD-L1 was obviously higher in xenografts formed by IL-17A-treated CD133$^+$ hepatoma cells than by control-treated CD133$^+$ cells (Fig. 4E-F; Fig. S4B). Consistent with this, the supernatant from the co-culture of lymphatic endothelial cells with CD133$^+$ cells, isolated from Huh-7 xenografts, induced the expression of PD-L1 in CD133$^+$ cells (Fig. S4C). Thus, IL-17A promotes PD-L1 expression in CD133$^+$ hepatoma stem cells.

Next, we examined the contribution of PD-L1 to the IL-17A-promoted resistance of CD133$^+$ hepatoma cells to CD8$^+$ T cells. Anti-PD-L1 antibody reduced the positive effect of IL-17A on the resistance of CD133$^+$ cells, isolated from Huh-7 xenografts, to CD8$^+$ T cells (Fig. 4G). Thus, IL-17A promotes the resistance of CD133$^+$ hepatoma stem cells to CD8$^+$ T cells through upregulation of PD-L1.

IL-17A secreted from lymphatic endothelial cells promoted self-renewal of CD133$^+$ hepatoma cells through upregulation of PD-L1

PD-L1 promotes the self-renewal of CSCs.[27] Supporting this notion, downregulation of PD-L1 by shRNA reduced sphere formation of CD133$^+$ hepatoma cells isolated from Huh-7 or PLC/PRF/5 xenografts (Fig. 5A-C; Fig. S5A), and reduced the tumor-initiating capacity of CD133$^+$ hepatoma cells isolated from Huh-7 or PLC/PRF/5 xenografts (Fig. S5B). PD-L1 could activate Akt signaling,[27,28] which promotes the self-renewal of CSCs.[29] PD-L1 downregulation reduced Akt phosphorylation in CD133$^+$ cells isolated from Huh-7 xenograft (Fig. S5C). Overexpression of the active form of Akt (myr-Akt) rescued the negative effect of PD-L1 downregulation on the sphere formation of CD133$^+$ hepatoma cells isolated from Huh-7 xenografts (Fig. S5D-E).

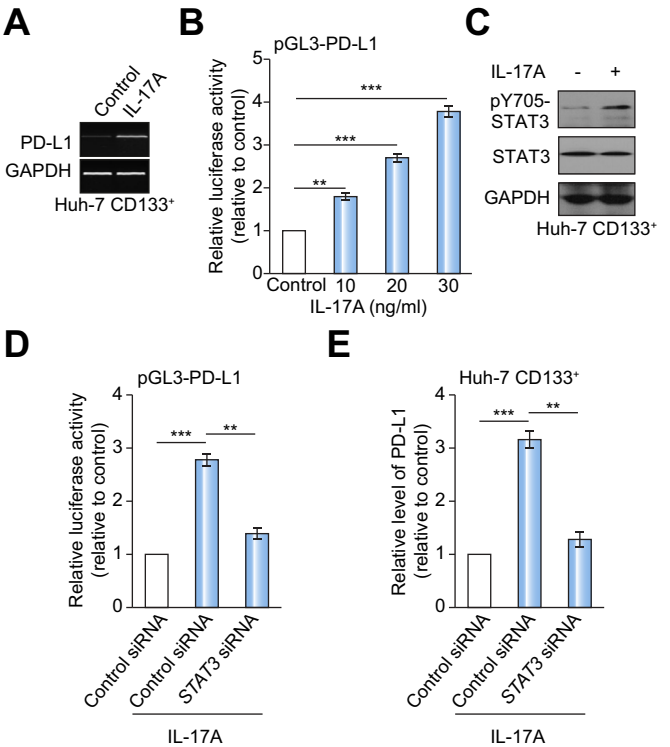


Fig. 6. IL-17A promoted *PD-L1* transcription through activation of STAT3 phosphorylation. (A) RT-PCR analysis of *PD-L1* mRNA level in CD133$^+$ cells isolated from Huh-7 xenograft treated with IL-17A. (B) CD133$^+$ cells isolated from Huh-7 xenograft transiently transfected with pGL3-*PD-L1* and pRL-SV40 were treated with an increased dose of IL-17A. Luciferase activity was determined and was standardized to that of CD133$^+$ cells treated with control. Results are expressed as mean ± SEM (n = 4). Data analyzed by ANOVA tests, $^{**}p$ <0.01, $^{***}p$ <0.001. (C) Western blot analysis of STAT3 Y705 phosphorylation in CD133$^+$ cells isolated from Huh-7 xenograft treated with IL-17A. (D) CD133$^+$ cells isolated from Huh-7 xenograft transiently transfected with pGL3-*PD-L1* and pRL-SV40 with or without *STAT3* siRNA were treated with IL-17A for 24 h. Luciferase activity was determined. Results are expressed as mean ± SEM (n = 4). Data analyzed by 2-tailed *t* tests, $^{**}p$ <0.01, $^{***}p$ <0.001. (E) qRT-PCR analysis was performed for PD-L1 in CD133$^+$ hepatoma cells isolated from Huh-7 xenograft expressing control siRNA or *STAT3* siRNA treated with IL-17A. Results are expressed as mean ± SEM (n = 4). Data analyzed by 2-tailed *t* tests, $^{**}p$ <0.01, $^{***}p$ <0.001. qRT-PCR, quantitative reverse transcription PCR.

Thus, downregulation of PD-L1 inhibits the self-renewal and tumorigenesis of CD133$^+$ hepatoma stem cells.

Next, the contribution of upregulation of PD-L1 by IL-17A in the self-renewal of hepatoma stem cells was evaluated. PD-L1 downregulation partly reduced the positive effect of IL-17A on sphere formation of CD133$^+$ hepatoma cells isolated from Huh-7 and PLC/PRF/5 xenografts (Fig. 5D). The supernatant from the co-culture of CD133$^+$ hepatoma cells isolated from HCC samples with lymphatic endothelial cells significantly promoted the sphere formation of CD133$^+$ cells, which was blocked by IL-17A downregulation in lymphatic endothelial cells (Fig. S5F), or PD-L1 downregulation in CD133$^+$ hepatoma cells (Fig. 5E). Together, IL-17A secreted from lymphatic endothelial cells promotes the self-renewal of CD133$^+$ hepatoma cells, partly through upregulation of PD-L1.

Next, we evaluated the contribution of IL-17A secreted from lymphatic endothelial cells to tumorigenesis of CD133$^+$ hepatoma cells. Co-injection with lymphatic endothelial cells promoted tumorigenesis of CD133$^+$ hepatoma cells isolated from Huh-7 xenografts, which was partly blocked by downregulated expression of IL-17A in lymphatic endothelial cells (Fig. S5G). VEGF-C stimulates lymphangiogenesis through binding to VEGFR3.[30,31] Soluble VEGFR3 extracellular domains containing Ig homology domains 1-3 captures VEGF-C and thus blocks VEGFR3 signal activity, subsequently reducing lymphangiogenesis.[32,33] CD133$^+$ hepatoma cells isolated from Huh-7 and PLC/PRF/5 xenografts were orthotopically co-injected with sVEGFR3-Fc and/or IL-17A into immunodeficient mice. Immunohistochemical analysis showed that sVEGFR3-Fc obviously reduced lymphangiogenesis *in vivo* (Fig. S5H). Compared to Fc, sVEGFR3-Fc reduced the tumor-initiating capacity of CD133$^+$ cells, isolated from Huh-7 and PLC/PRF/5 xenografts, which could be partly rescued by IL-17A protein (Fig. 5F-G). Together, IL-17A secreted from lymphatic endothelial cells promotes CD133$^+$ hepatoma stem cell tumorigenesis.

IL-17A promoted PD-L1 transcription through activation of STAT3 phosphorylation

IL-17A increased the level of *PD-L1* mRNA (Fig. 6A). Consistent with this, IL-17A increased the activity of the *PD-L1* promoter in a dose- and time-dependent manner (Fig. 6B and Fig. S6A). It has been reported that STAT3 binds to the *PD-L1* promoter and activates *PD-L1* transcription.[34] IL-17A activates the JAK/STAT3 signaling pathway.[35] IL-17A increased STAT3 Y705 phosphorylation in CD133$^+$ cells isolated from Huh-7 xenografts (Fig. 6C). Downregulation of STAT3 blocked IL-17A-induced *PD-L1* promoter activity (Fig. 6D), and reduced IL-17A-induced *PD-L1* mRNA levels in CD133$^+$ cells isolated from Huh-7 xenografts (Fig. 6E). Thus, IL-17A promotes *PD-L1* transcription through activation of STAT3 phosphorylation.

IL-17A was highly expressed in hepatoma and IL-17A expression overlapped with PD-L1 expression

Next, we investigated the characteristics of IL-17A expression during hepatoma progression. Compared to para-tumor tissues, IL-17A was highly expressed in hepatoma (Fig. 7A-B; p <0.001). Immunohistochemical staining for IL-17A in hepatoma tissues showed the diffusive expression of IL-17A in hepatoma (Fig. 7C). Interestingly, IL-17A was partially expressed in single cells (Fig. 7D) or cells surrounding capillaries (Fig. 7E). Considering that IL-17A promotes PD-L1 expression, the mRNA expression of *IL-17A* and *PD-L1* in hepatoma samples was analyzed

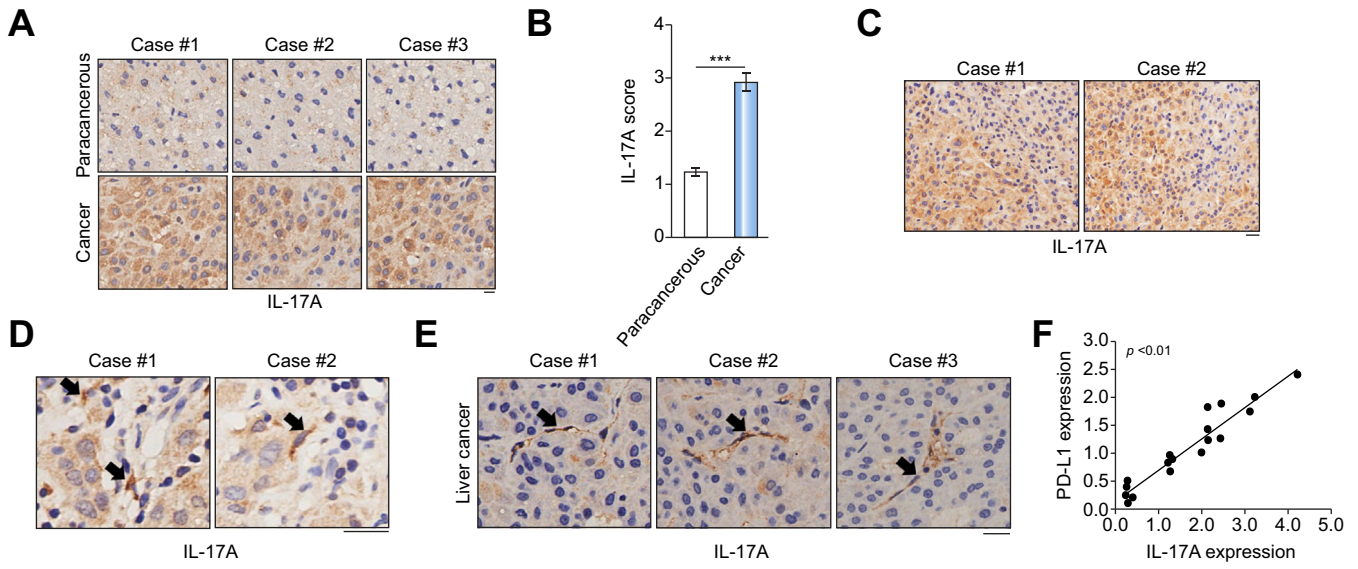


Fig. 7. IL-17A was highly expressed in hepatoma and its expression was correlated with PD-L1 expression. (A-B) Immunohistochemical staining for IL-17A in hepatoma tissues and paracancerous tissues. (A) Representative images are shown. Scale bar, 10 μM. (B) The scores for quantitative staining of IL-17A in hepatoma tissues and paracancerous tissues were determined. Results are expressed as mean ± SEM (n = 40) Data analyzed by 2-tailed *t* test, ***p <0.001. (C) Immunohistochemical staining for IL-17A in hepatoma tissues showed the diffusive expression of IL-17A in hepatoma. Scale bar, 20 μM. (D-E) Immunohistochemical staining for IL-17A in hepatoma tissues showed that IL-17A was partially expressed in single cells (D, arrow) or cells surrounding capillaries (E, arrow). Scale bar, 20 μM. (F) The correlation of IL-17A expression with PD-L1 expression in hepatoma tissues was analyzed using Spearman's r test (n = 20, **p <0.01). (This figure appears in colour on the web.)

using qRT-PCR. The *IL-17A* and *PD-L1* mRNA levels exhibited a strong positive correlation in hepatoma tissues (Fig. 7F; R^2 = 0.9086, p <0.01).

Inhibition of IL-17A signaling pathway reduced the self-renewal ability and tumorigenesis of CD133$^+$ hepatoma cells

The contribution of IL-17A to hepatoma stem cells self-renewal motivated us to explore the effects of a IL-17 pathway inhibitor on the self-renewal of hepatoma stem cells. Fc-fused proteins containing member protein extracellular domain are widely used to inhibit member protein interaction with its ligand.[36,37] The extracellular domain of the IL-17A receptor was fused to the N-terminal region of Fc containing mouse IgG CH2 and CH3 domain (IL-17RA-Fc-6×His, Fig. 8A). IL-17RA-Fc interacted with IL-17A (Fig. 8B) and reduced the binding of IL-17A to CD133$^+$ hepatoma cells isolated from Huh-7 xenografts (Fig. 8C). Thus, IL-17RA-Fc effectively reduces IL-17A signaling. Compared to control, IL-17RA-Fc significantly reduced the positive effect of IL-17A on sphere formation of CD133$^+$ hepatoma cells isolated from Huh-7 xenografts (Fig. 8D-E). Furthermore, injection of IL-17RA-Fc protein reduced the growth *in vivo* of CD133$^+$ hepatoma cells isolated from Huh-7 xenografts (Fig. 8F). Together, inhibition of IL-17A signaling pathway by IL-17A receptor fusion protein reduces the self-renewal and tumorigenesis of CD133$^+$ hepatoma stem cells.

Discussion

Herein, we have shown that the interaction between the mannose receptor and high-mannose type glycans mediated the physical interactions between hepatoma stem cells and lymphatic endothelial cells. This interaction activated cytokine *IL-17A* transcription and secretion. IL-17A promoted self-renewal of hepatoma stem cells and their immune escape (Fig. 8G).

The interactions between lectin and glycan regulate the cell microenvironment.[38] Here, we have found that the structure of N-glycans in hepatoma stem cells was mainly high-mannose type N-glycans. Treatment with Con A lectin recognizing high-mannose type N-glycans or mannose significantly reduced the adherence of CD133$^+$ hepatoma stem cells to lymphatic endothelial cells. Thus, high-mannose type N-glycans on hepatoma stem cells are required for their interaction with lymphatic endothelial cells. However, the mechanism of high-mannose type N-glycan formation in hepatoma stem cells needs to be investigated further.

IL-17A is mainly produced by CD4$^+$ T cells and/or γδ T cells in a variety of tumors.[19,39] We also showed that the expression of

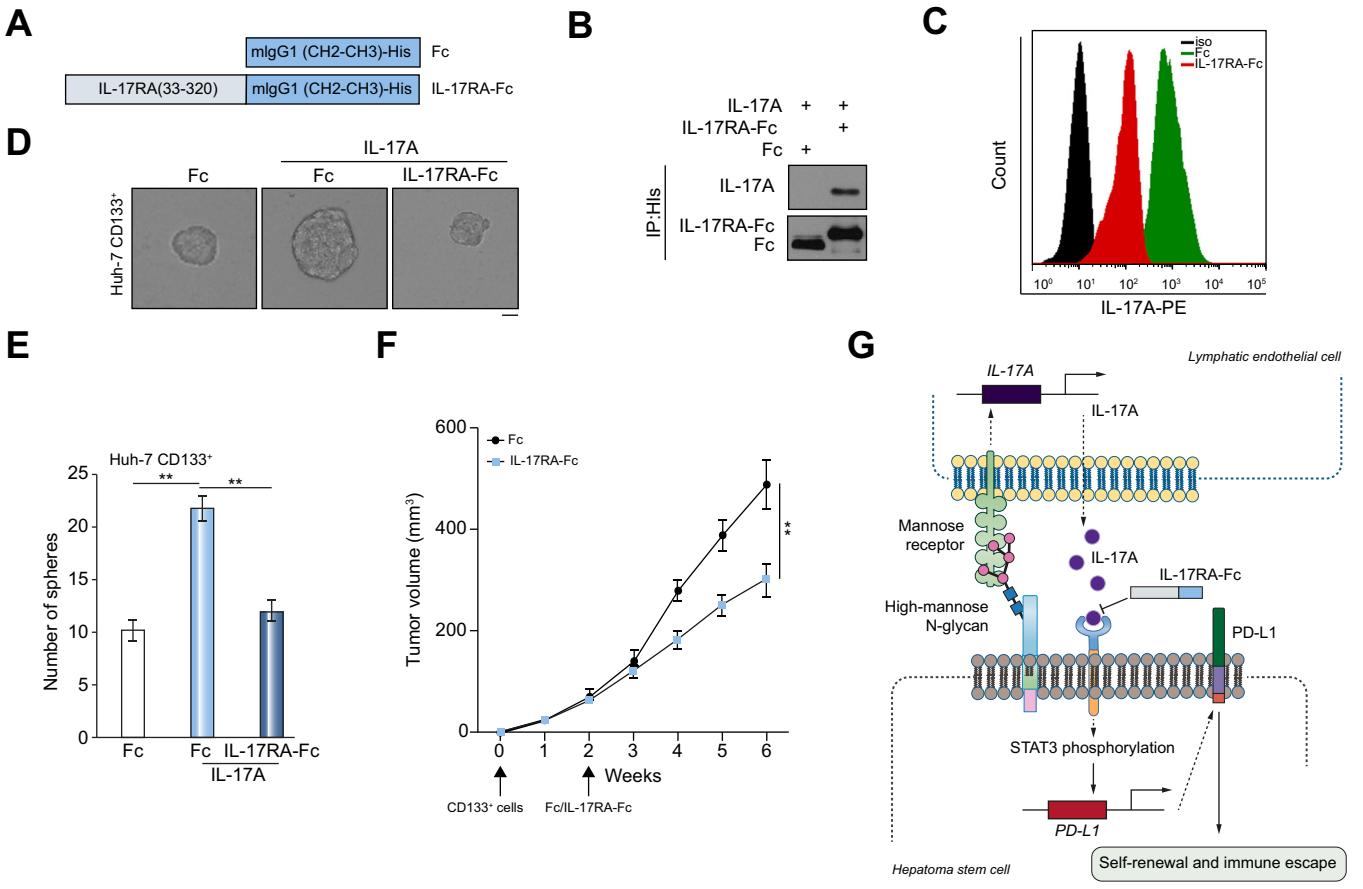


Fig. 8. Inhibition of IL-17A signaling pathway reduced the self-renewal capacity and tumorigenesis of CD133$^+$ hepatoma stem cells. (A) Schematic of fusion protein. (B) *In vitro* interaction between IL-17RA-Fc and IL-17A. (C) Flow cytometry analysis of the binding of PE-conjugated IL-17A to CD133$^+$ cells isolated from Huh-7 xenograft pretreated with IL-17RA-Fc. (D-E) Sphere formation assay of CD133$^+$ hepatoma cells isolated from Huh-7 xenograft treated with IL-17A and Fc or IL-17RA-Fc. (D) Representative images of sphere are shown. Scale bar, 10 μM. (E) Results are expressed as mean ± SEM (n = Data analyzed by 2-tailed *t* tests, $^{**}p$ <0.01. (F) CD133$^+$ cells isolated from Huh-7 xenograft were subcutaneously co-implanted into immunocompromised mice. After 14 days, mice were injected with Fc or IL-17RA-Fc. Tumor volumes were measured after tumor cell inoculation every week. Results are expressed as mean ± SEM (n = 6 mouse). Data analyzed by 2-tailed *t* tests, $^{**}p$ <0.01. (G) Model of interaction between lymphatic endothelial cell and hepatoma stem cell. (This figure appears in colour on the web.)

IL-17A in lymphatic endothelial cells was obviously lower than in $CD4^+$ T cells. However, the mannose receptor was highly expressed in lymphatic endothelial cells. IL-17A from lymphatic endothelial cells promoted self-renewal of hepatoma stem cells and consequently tumorigenesis. However, the effects of IL-17A from T cells on hepatoma stem cell behaviors need to be investigated further.

Tumors utilize many different mechanisms to promote immune escape.[40] PD-1 and its ligand PD-L1 play a key role in tumor immune escape.[41] IL-17A promotes immune escape of hepatoma stem cells at least partly through upregulation of PD-L1 expression. Thus, the interactions between lymphatic endothelial cells and CSCs promote hepatoma stem cells immune escape in a paracrine manner.

CSCs maintain their characteristics in a specific microenvironment. Therefore, microenvironment-targeting strategies that could eliminate the CSC population might improve the clinical outcomes of patients with cancer. Our findings have shown that neutralization of IL-17A signaling inhibited the self-renewal of hepatoma stem cells. Thus, inhibition of IL-17A signaling may be a promising approach for hepatoma treatment.

In summary, our results prove that lymphatic endothelial cells build a hepatoma stem cell niche. IL-17A secreted from this niche promotes the self-renewal, tumorigenesis and immune escape of hepatoma stem cells. These findings identify lymphatic endothelial cells as an important component of the hepatoma stem cell niche.

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Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Yuanyan Wei, Danfan Shi, Ziwei Liang, Yuming Liu, Yang Xing, Yinan Li, Weitao Liu, Xiaoning Chen performed the experiments. Zhilong Ai and Jianhui Zhuang analyzed data. Qiang Gao and Yuming Liu provided hepatoma tissues. Yuanyan Wei and Jianhai Jiang designed experiments. Yuanyan Wei and Jianhai Jiang wrote manuscript. All authors approved the final version of the manuscript.

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2019.08.034.

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Author names in bold designate shared co-first authorship

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