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The therapeutic effect of IL-21 combined with IFN-γ inducing $CD4^+CXCR5^+CD57^+$T cells differentiation on hepatocellular carcinoma

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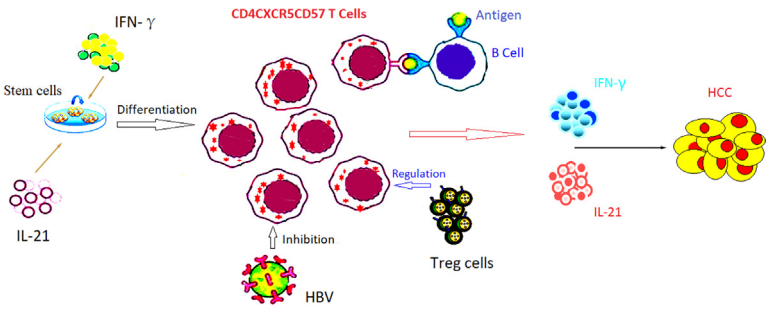
HIGHLIGHTS

- In patients with HCC with prolonged survival time, the number of $CD4^+CXCR5^+CD57^+$T cells increased significantly.
- IL-21 combined with INF-γ induce stem cells to differentiate into $CD4^+CXCR5^+CD57^+$T cells, which can induce apoptosis of HepG2 cells.
- HBV inhibits the number and function of $CD4^+CXCR5^+CD57^+$T cells.
- Treg cells regulates $CD4^+CXCR5^+CD57^+$T cells.
- $CD4^+CXCR5^+CD57^+$T cells induced by IL-21 combined with INF-γ can inhibit the growth of liver cancer.

GRAPHICAL ABSTRACT



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ABSTRACT

Introduction: Liver cancer is a malignant tumor with high incidence and short survival time. In order to increase the cure rate and disease-free survival rate of liver cancer, it is necessary to seek effective treatment methods.
Objectives: The objective of this study is to evaluate the therapeutic effects of IL-21 and IFN-γ inducing the formation of $CD4^+CXCR5^+CD57^+$T cells on liver cancer.
Methods: The methods of analyze the relationship between $CD4^+CXCR5^+CD57^+$T cells and the survival time of hepatocellular carcinoma (HCC), and study the effect of IL-21 combined with IFN-γ in inducing stem cells to differentiate into $CD4^+CXCR5^+CD57^+$T cells. The effects of IL-21 combined with IFN-γ induced $CD4^+CXCR5^+CD57^+$T cells on liver cancer were studied through animal experiments, and the regulatory mechanism, and the effect of hepatitis B virus (HBV) on it.
Results: The study found that the number of $CD4^+CXCR5^+CD57^+$T cells in serum of liver cancer patients with prolonged survival time increased significantly, the expression of CD4, CD57, and CXCR5 in the tumor microenvironment increased, and the serum IL-21 and IFN-γ concentrations increased. IL-21 and IFN-γ induce stem cells to differentiate into $CD4^+CXCR5^+CD57^+$T cells and induce HepG2 cells apoptosis. HBV leads to a decrease in the number of $CD4^+CXCR5^+CD57^+$T cells and a chronic inflammatory response. Treg cells can regulate $CD4^+CXCR5^+CD57^+$T cells. IL-21 combined with IFN-γ induced an increase in the number of $CD4^+CXCR5^+CD57^+$T cells in hepatocarcinoma-bearing mice, which has an inhibitory effect on H22 liver cancer.
Conclusion: The conclusion of the study is that IL-21 combined with IFN-γ induces stem cells to differentiate into $CD4^+CXCR5^+CD57^+$T cells, Treg can control the increase in their number, and HBV can cause their number to decrease, which can control the growth of liver cancer.

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Introduction

In 2018, the incidence of liver cancer in China was 392,868, accounting for 9.2% of malignant tumors, and the incidence was the third; the death toll was 368,960, accounting for 12.9% of the total cancer deaths, and the mortality was the third [1]. In most countries, the mortality rate of liver cancer is almost equal to the morbidity rate, indicating the lack of effective treatments. The risk factors of liver cancer mainly include hepatitis B or C infection and alcoholic liver cirrhosis [2]. Liver cirrhosis and liver dysfunction are direct prognostic factors [3]. In order to increase the cure rate and disease-free survival rate of liver cancer, it is necessary to seek effective treatment methods.

Follicular helper T cells (Tfh) play a key role in the production of B lymphocyte antibodies. Tfh cells express CXCR5 and produce interleukin-21 (IL-21) and interleukin-6 (IL-6) [4]. When autoimmune diseases occur, Tfh migrates to the injured site, produces IL-21 and lymphocyte infiltration, and makes B cells produce high-affinity antibodies [5]. Tfh cells can over-activate B cells and produce a large amount of immunoglobulin [6,7], while interleukin-2 (IL-2) can inhibit the maturation of Tfh in the germinal center and hinder the differentiation of B cells [8]. The lack of Roquin protein will destroy the inhibitory effect of Treg cells, upregulate the expression, and increase the number of Tfh cellular genes [9]. In the presence of IL-21 but not TGF-β, primitive T cells activated in vitro preferentially express the Tfh gene and promote the germinal center response in vivo study [10].

The increase in the number of $CXCR5^+CD4^+$T cells in patients with chronic hepatitis B is related to ALT and AST levels [11], but has nothing to do with HBV-DNA levels and IL-21R gene expression [12]. The effector molecules of Tfh cells can be used as effective therapeutic targets for systemic lupus erythematosus [13]. Tfh cell dysfunction, high or low function, increase or decrease of ICOS or IL-21 are closely related to autoimmune diseases or immunodeficiency diseases [14]. $CD57^+$T cells are produced in bone marrow and liver and are closely related to tumor immunity [15]. CD57 expressing lymphocytes show high potential cytotoxicity, have similar memory characteristics and powerful effector functions. The frequency of CD57 expression by cells in blood and tissues is related to the clinical prognosis of chronic infections or various cancers and human aging. $CD57^+$T cells mainly secrete interferon-γ (IFN-γ). As a new strategy against human immune aging and/or various chronic diseases [16]. CD57 and Tfh cells have a certain correlation. $CD57^+$T cells are located in the germinal center, and the germinal center also expresses CXCR5, but its specific role is not clear.

In the study, IL-21 and IFN-γ induce the formation of $CD4^+$-$CXCR5^+CD57^+$T cells and their therapeutic effects on liver cancer.

Materials and method

Patient samples

This study is a single-center study of the relationship between $CD4^+CXCR5^+CD57^+$ T cells and the overall survival (OS) of liver cancer. This study is divided into control group, liver cancer stage I-II group and stage IV group. Inclusion criteria included the patient was diagnosed as hepatocellular carcinoma through histology, and intrahepatic cholangiocarcinoma and hepatosarcoma were excluded. The stage of the patient's disease is confirmed by CT or MRI, and the distant metastasis needs to be confirmed by CT, MRI or bone scan. The disease staging is carried out according to the AJCC (2010) liver cancer staging. The enrolled patients were all diagnosed for the first time and had not undergone surgery, interventional therapy, targeted therapy, chemotherapy and immunotherapy. The enrollment age is 18–70 years old. Complicated diseases such as lung infection and cholecystitis were excluded at the time of enrollment. The control group consisted of 30 normal people, aged 18–70 years old, excluding chronic hepatitis B or C, chronic eczema, asthma, and autoimmune diseases such as systemic lupus erythematosus etc.

Sample. The venous blood of the patient and the venous blood of the control group were taken for testing at the first diagnosis. Pathological specimens take the specimens from the patient's first operation or the pathological specimens from liver biopsy for testing. Identification of $CD4^+CXCR5^+CD57^+$T cells and the inhibitory effect of IFN-γ and IL-21 on liver cancer. The test method included detecting the concentration of serum IL-6, IL-21, IFN-γ, and CXCR5 in patients with liver cancer, and detecting the number of $CD4^+$-$CXCR5^+CD57^+$T cells in the peripheral blood by flow cytometry. To analyze the correlation between the OS of liver cancer patients and $CD4^+CXCR5^+CD57^+$T cells.

The relationship between ALT, IgG, HBV-DNA, and $CD4^+CXCR5^+$-$CD57^+$T cells. Liver cancer specimens were fixed with 4% paraformaldehyde, hematoxylin-eosin staining, pathological observation, and immunohistochemical (IHC) detection of CD57, HbeAg, CD4, CXCR5. Detect the amount of HBV-DNA in the blood. Evaluate the relationship between these indicators and the number of $CD4^+CXCR5^+CD57^+$T cells.

Ethics statement

All patients provided written informed consent. The study was approved by the ethics committee of Guangdong Pharmaceutical University, China (approval no. GDPU-2015110). All patients provided written informed consent according to Good Clinical Practice (GCP) and national regulations.

Cell culture and reagents

HepG2 cell line purchased from Guangdong Medical Experimental Animal Center, license number SCXK (Guangdong) 2013–0034. The cells were cultured in 1640 medium with 10% fetal bovine serum and penicillin. Stem cells extracted from peripheral blood donated by normal people. Stem cell collection method, take fresh anticoagulant blood and mix it with tissue diluent in a ratio of 1:1, and settle naturally at 30 °C for 30 min. Take the supernatant and mix with 1:1 cell washing solution, centrifuge at 500g for 15 min, discard the supernatant and add the diluent to suspend precipitate the cells, carefully superimpose the suspension on the surface of the cell separation solution, centrifuge at 400g for 20 min, collect the second layer of ring-shaped milky white cells, put it into 10 times the volume of the cell washing solution, mix well and add 500g Centrifuge for 15 min, which is the desired stem cells.

IFN-γ and IL-21 induce differentiation of $CD4^+CXCR5^+CD57^+$T cells. The Ficoll method was used to isolate stem cells from the peripheral blood of normal subjects. On the first day, IFN-γ, IL-21, IL-2 and IL-1 medium were added for culture at a concentration of IFN-γ (1000U/ml), CD3McAb (50 ng/ml), IL-2 (500U/ml), IL-1α (100U/ml), IL-21 (50 ng/ml). The cells proliferate about 30 ~ 50

times, and routine cell culture starts on the third day. The number of $CD4^+CXCR5^+CD57^+$T cells was detected by flow cytometry on the 4th, 7th, and 12th day. ELISA detected the concentration of IL-21, IL-6, IFN-γ, and CXCR5 on the 12th day after $CD4^+CXCR5^+CD57^+$T cells culture.

$CD4^+CXCR5^+CD57^+$T cells induced HepG2 cells apoptosis. Add 0.5 ml of $CD4^+CXCR5^+CD57^+$T cell culture medium on the 12th day to the HepG2 cell culture medium, which accounts for 10% of the total culture medium volume. Detect HepG2 cells apoptosis in 24 ~ 48 h, and detect by flow cytometry FasL expression; Annexin V-FITC/PI double stained cells were used to detect apoptosis.

Animal model

C57BL/6.Cg-Tg(HBV) Smoc mice were purchased from Shanghai Southern Model Biology Research Center, $FOXP3^{-/-}$C57BL/6J mice were purchased from Jackson Labs, and C57BL/6J mice were purchased from Guangdong Medical Experimental Animal Center. C57BL/6.Cg-Tg(HBV) Smoc mice, transfers 1.0 copy of the full-length DNA of c1 type HBV virus into mice. The transgenic mice were positive for HBsAg in the serum, but had no expression of HBeAg, anti-HBs, anti-HBe or antiHBc. The stably expressed human hepatitis B virus surface antigen can be detected in the serum, liver and kidney of the transgenic mouse model.

The inhibitory effect of HBV infection on $CD4^+CXCR5^+CD57^+$T cells. Use flow cytometry to detect the number of peripheral blood $CD4^+CXCR5^+CD57^+$T cells in C57BL/6J.Cg-Tg(HBV)Smoc and C57BL/6J mice. Elisa detects the concentration of serum IL-21, IL-6, CXCR5, andCD57. HE staining analyzes the pathological changes of the liver and lung.

The regulatory effect of Treg cells on $CD4^+CXCR5^+CD57^+$T cells. Use flow cytometry to detect the number of peripheral blood $CD4^+$-$CXCR5^+CD57^+$T cells in $FOXP3^{-/-}$C57BL/6J and C57BL/6J mice. Elisa detects the concentration of serum IL-21, IL-6, CXCR5, and CD57. HE staining to analyze the pathological changes of liver and lung and IHC to analyze CD4, CXCR5, and CD57 in the liver.

The therapeutic effects of IFN-γ and IL-21 on liver cancer through $CD4^+CXCR5^+CD57^+$T cells. C57BL/6J mice, male and female, 4–6 weeks, divided into model group and treatment group. Establishment of H22 liver cancer transplantation tumor model. H22 cells count, 1×10^7/ml, 0.1 ml was injected subcutaneously into the left chest, and transplanted tumors formed after 3 days.

In the treatment group, IL-21 and IFN-γ were intervened. H22 transplanted tumor mice were intraperitoneally injected with IL-21 1U/g and IFN-γ 1U/g for 3 consecutive days. After 14 days, detect the changes of serum IL-21, IL-6, CXCR5, CD57, and CXCL13, the changes of spleen $CD4^+CXCR5^+CD57^+$ T cells, and the proteins of IL-21, IL-6, CXCR, CD57 in H22 transplanted tumors. H22 xenograft

tumor was tested for Changes of IL-21, IL-6, CXCR5, and CD57 genes by Quantitative real-time PCR.

Ethics statement

All experiments involving animals were conducted according to the ethical policies and procedures approved by the ethics committee of Guangdong Pharmaceutical University, China (approval no. GDPU-2015110).

Experimental method

Flow cytometry

Cut the spleen and separate lymphocytes. Add about 2 ml of erythrocyte lysis buffer and lyse for 2 min. Take 30 ml of the lymphocyte separation solution, slowly add the lysed suspension cells to the lymphocyte separation solution and centrifuge. Centrifuge the middle layer of lymphocytes for 15 min. Use 1640 culture medium to adjust to 2×10^5/ml. Add 4 μl each of PMA IIonomycin and Monensin IBFA. Incubate at 37 °C for 6 h. Add CD57 FITC 1.25 μl, CD4 PE-A 1.25 μl, and CXCR5 PE-Cy7-A 1.25 μl to each sample. The detection control group was added with CD90-FITC and incubated at room temperature for 30 min in the dark. Take CD4 as the door and test on the machine. CD4, CD57 and CXCR5 were purchased from EBioscience, Inc.

Quantitative real-time PCR

Total RNA was extracted from Liver cancer specimens or xenografts tumor. Quantitative Real-time PCR was set up with ABI Power SYBR Green PCR Master Mix (ABI, USA) and synthetic probe of Shanghai Shengong Bioengineering Company. PCR reaction was performed on the 7900 HT Sequence Detection System (ABI, USA) followed these conditions: step 1 (denaturation) at 95 °C for 5 min, step 2 (amplification) at 60 °C for 30 min, step 3 (cooling) at 40 °C for 30 sec. The expression of each gene was normalized to housekeeping gene actin. (Table 1).

IHCxxx

The transplanted tumor is deparaffinized and the antigen is repaired. Add the primary antibody and PBS to the control tissue. Add secondary antibody, wash with distilled water and color reagent for 5 min. After rinsing in clean water, soak in hematoxylin for 30 s. Washed to differentiate and back to blue. Dehydrate and analyze under a microscope.

ELISAxxx

Dilute the standard. Add 40 μl of sample diluent to the sample well, and then add 25 μl of the sample to be tested. Incubate at 37 °C for 30 min. After washing, add 50 μl of enzyme-labeled reagent, incubate at room temperature, develop color, add 50 μl of stop solution, and measure the absorbance (OD value) of each well at 450 nm wavelength.

Fluorescence quantitative PCR

Hepatitis B virus nucleic acid quantitative detection kit (Shanghai Kehua Biological Engineering Co., Ltd.). Take a 40 μl serum sample, add the same amount of DNA extract, and mix well. Amplify on a PCR amplifier and determine the results; the amplification conditions are 37 °C for 5 min, 94 °C for 1 min, 95 °C for 5 s, 60 °C for 30 s, 40 cycles, and a reaction volume of 40 μl. As a result, the reagent sensitivity was 5.0×10^2 copise/ml. Result judgment, the logarithm of the gene copy concentration of the 4 positive control substances was the abscissa, and the actual measured *Ct* value was used as the ordinate to make the standard curve. The fit R of the standard curve should be greater than or equal to 0.990; otherwise, it is invalid.

Table 1
Primer sequence.

IL-21	Forward	Primer	TTGCACAGCAGTCTTGAACC
IL-21	Reverse	Primer	GCTTACACCAGTGGCAACCT
IL-6	Forward	Primer	TCCTAACAGATAAGCTGGAGTCACA
IL-6	Reverse	Primer	TGCCGAGTAGATCTCAAAGTGACTT
CXCR5	Forward	Primer	CCAGGCTCTAAGGAAGAACAAGTAAA
CXCR5	Reverse	Primer	CCCCACCAGTCTGTAAGCTTTG
CD57	Forward	Primer	AACCCCGCTCCCTCGAT
CD57	Reverse	Primer	GCCCATGGTGCTCCTGAA
Actin	Forward	Primer	TGGCTCCTAGCACCATGAAGA
Actin	Reverse	Primer	GCCACCGATCCACACAGAGT

Statistical analysis

The experimental results are analyzed by SPSS22.0 statistical software, and the data are expressed as mean ± standard deviation (x ± s). If measurement data conform to normal distribution and homogeneity of variance, use independent sample *t* test or one-way analysis of variance LSD method. For normal distribution or uneven variance, the Wilcoxon rank sum test was used for statistical analysis. Flow data is analyzed using the FlowJo software V10. Use GraphPad Prism 8.2.1 software for cytokine statistical analysis. Take $P < 0.05$ as the standard of statistical significance.

Results

In patients with HCC with prolonged survival time, the number of $CD4^+CXCR5^+CD57^+$T cells increased significantly, and the concentration of IL-21 and IFN-γ in the serum increased

The enrollment of this trial case was from April 2015 to April 2019. There were 60 HCC patients, 28 of which were stage I-II while 32 stage IV. The pathological diagnosis was HCC. The clinical parameters related to HCC were summarized in Table 2. HBV-DNA is positive with > 5.00E + 02 IU/ml. BMI ≥ 28.0 is the standard for obesity. Drinking status, men drink no more than 25 g of alcohol a day, and women no more than 15 g.

$CD4^+CXCR5^+CD57^+$T cell detection results. Compared with the normal population, patients with stage I-II liver cancer and stage IV liver cancer showed a decrease in $CD4^+CXCR5^+CD57^+$T cells, while the decrease in stage IV patients was more obvious (Fig. 1A). In stage IV HCC, the number of $CD4^+CXCR5^+CD57^+$T cells decreased compared with the normal population (Fig. 1B). Concentration of serum IL-21. Compared with the normal population, the serum IL-21 concentration detected by Elisa showed a decrease in the liver cancer population. The later the disease stage, the more obvious the decrease (Fig. 1C). Concentration of serum IFN-γ. The concentration of serum IFN-γ decreased in patients with liver cancer, and decreased more significantly in patients with stage IV (Fig. 1D). Serum concentration of IL-6. The concentration of serum IL-6 in patients with liver cancer, the concentration of IL-6 increases, which is statistically significant. It shows that IL-6 has no correlation with $CD4^+CXCR5^+CD57^+$T cells (Fig. 1E).

The relationship between the survival time of HCC patients and the number of $CD4^+CXCR5^+CD57^+$T cells. The survival analysis of liver cancer patients shows that the more $CD4^+CXCR5^+CD57^+$T cells, the longer the survival time of patients (Fig. 1F). The results of the study showed that the more $CD4^+CXCR5^+CD57^+$T cells in HCC patients, the longer the patient OS.

Clinical studies have shown that the number of $CD4^+CXCR5^+$-$CD57^+$T cells in stage I-II HCC was higher than that in stage IV HCC, and the concentration of IL-21 and IFN-γ is higher than that in stage IV HCC, indicating the number of $CD4^+CXCR5^+CD57^+$T cells subsets decreased in advanced HCC. Survival analysis showed that in patients with stage IV HCC, $CD4^+CXCR5^+CD57^+$T cells have significant anti-liver cancer effects and can delay the progression of liver cancer.

$CD4^+CXCR5^+CD57^+$T cells lead to increased IgG and ALT

The factors related to $CD4^+CXCR5^+CD57^+$T cells. In the detection of 32 patients with stage IV liver cancer, it was found that when immunoglobulin G (IgG) increased, the number of $CD4^+CXCR5^+$-$CD57^+$T cells increased significantly, and there was a significant correlation between the two factors (Fig. 2A). When the number of $CD4^+CXCR5^+CD57^+$T cells increases, the serum ALT increases, there is a correlation between the two factors (Fig. 2B). It shows

Table 2
Clinical data.

Characteristic	No. of patients Total cases (n = 60)	$CD4^+CXCR5^+CD57^+$ T cells < 6%	$CD4^+CXCR5^+CD57^+$ T cells ≥ 6%	*P* value
Age (mean ± S.D.)	46.7 ± 12.6	44.3 ± 11.3	47.5 ± 12.2	0.616
Gender				0.735
Male	42	19	23	
Female	18	9	9	
Pathology				0.443
HCC	60	28	32	
Tumor size (cm)				0.024
T1	5	3	7	
T2	13	6	14	
T3	13	6	7	
T4	29	13	4	
Lymph node				0.015
N0	15	6	13	
N1	6	3	10	
N2	15	7	5	
N3	24	12	4	
Seroperitoneum	24	11	2	0.002
Distant metastasis	20	9	4	0.065
Clinical stage				0.001
I	8	1	7	
II	20	5	15	
III	12	6	6	
IV	20	16	4	
HBV-DNA IU/ml				
>5.00E+02	38	18	20	0.225
<5.00E+02	22	12	10	
Liver cirrhosis				
No	35	15	20	0.331
Yes	25	13	12	
Obesity				
No	48	23	25	0.476
Yes	12	5	7	
Drinking status				0.075
No	52	22	30	
Yes	8	6	2	

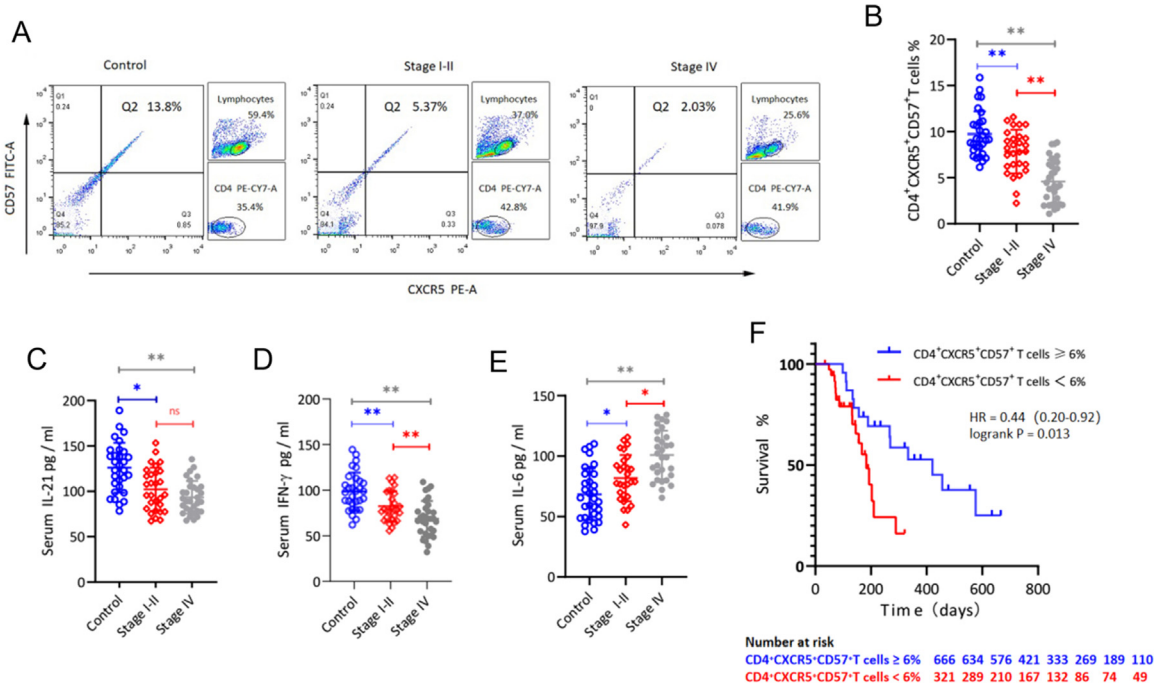

Fig. 1. $CD4^+CXCR5^+CD57^+$T cells prolong the survival time of liver cancer patients. (**A, B**) The normal population was 9.75 ± 2.46%, (n = 30), and the patients with stage I-II HCC were 7.87 ± 2.40% (n = 28, $^{**}P < 0.01$ vs control group). while the stage IV HCC was 4.65 ± 2.34% (n = 32, $^{**}P < 0.01$ vs control group, $^{**}P < 0.01$ vs stage I-II HCC). (**C**) The serum IL-21 concentration of patients with stage I-II HCC was 103.12 ± 23.77 pg/ml (n = 28), and that of the control group was 126.0 ± 27.45 pg/ml (n = 30, $^{*}P < 0.05$ vs stage I-II HCC). In stage IV HCC, it was 93.47 ± 17.92 pg/ml, (n = 32, $^{**}P < 0.01$ vs control group, $P = 0.112$ vs stage I-II HCC). **D** The concentration of serum IFN-γ in patients with stage I-II HCC was 82.47 ± 16.88 pg/ml (n = 28), and that of the control group was 98.72 ± 20.77 pg/ml (n = 30, $^{**}P < 0.01$ vs stage I-II HCC). In stage IV HCC, it was 69.48 ± 19.30 pg/ml (n = 32, $^{**}P < 0.01$ vs control group, $^{**}P < 0.01$ vs stage I-II HCC). **E** The serum IL-6 concentration of patients with stage I-II HCC was 81.98 ± 19.39 pg/ml (n = 27), and that of the control group was 67.97 ± 21.47 pg/ml (n = 30, $^{*}P < 0.05$ vs stage I-II HCC). In stage IV HCC, it was 100.95 ± 20.03 pg/ml (n = 32, $^{**}P < 0.01$ vs control group, $^{*}P < 0.05$ vs stage I-II HCC). **F** Among 60 patients with liver cancer, 32 cases had $CD4^+CXCR5^+CD57^+$T cells exceeding 6%, and 28 cases had lower than 6%. Taking 6% as the dividing line, the survival time of>6% is significantly longer than the number of people under 6% ($p = 0.039$). *<0.05, **<0.01, ns indicates normal.

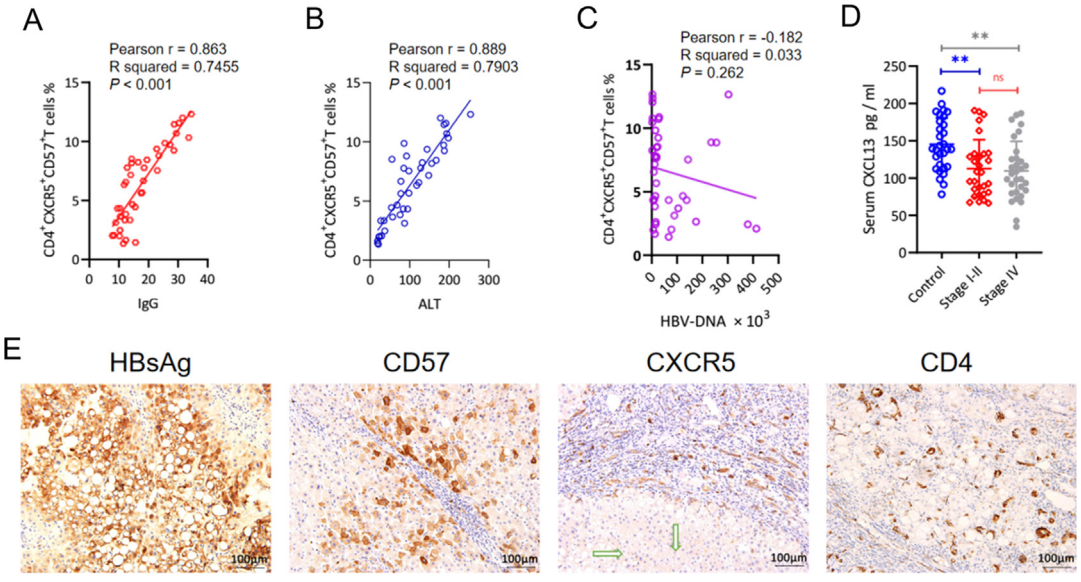

Fig. 2. $CD4^+CXCR5^+CD57^+$T cells related factors. (**A**) There is a significant correlation between $CD4^+CXCR5^+CD57^+$T cells and IgG in stage IV liver cancer (n = 32, $p < 0.001$). (**B**) There is a significant correlation between $CD4^+CXCR5^+CD57^+$T cells and ALT in stage IV liver cancer (n = 32, $p < 0.001$). (**C**) There is no correlation between HBV-DNA copy number in stage IV liver cancer (n = 32, $p > 0.05$). (**D**). The concentration of serum CXCL13 in the normal population is 145.33 ± 35.36 pg/ml (n = 30), and the liver cancer stage I-II was 110.44 ± 37.14 pg/ml, (n = 28, $^{**}p < 0.01$ vs normal population). The stage IV liver cancer was 110.68 ± 39.63 pg/ml, (n = 32, $^{**}P < 0.01$ vs control group, ns = 0.761, $P > 0.05$ vs stage I-II HCC). **E** IHC analysis of liver cancer showed that HbsAg was strongly positive, and CD4, CXCR5, and CD57 were all positively expressed. The CD4 arrow indicates normal liver cells. *<0.05, **<0.01, ns indicates normal.

that the increase in the number of $CD4^+CXCR5^+CD57^+$T cells can increase the body immune response. And the copy number of HBV-DNA has no correlation with $CD4^+CXCR5^+CD57^+$T cells (Fig. 2C). CXCL13 is a ligand for Tfh cells, although the concentration of CXCL13 decreased in liver cancer, there was no difference between stage I-II and stage IV liver cancer, indicating that there was no correlation with $CD4^+CXCR5^+CD57^+$T cells (Fig. 2D). Detect the expression of HbsAg, CD4, CXCR5, and CD57 in liver cancer specimens. The results showed that in liver cancer specimens, HbsAg was strongly positive, CD4, CXCR5, and CD57 were all positively expressed. In CD4 positive specimens, no CD4 expression was found in normal liver tissues (Fig. 2E).

IL-21 and IFN-γ induce stem cells to differentiate into $CD4^+CXCR5^+CD57^+$T cells, which can induce apoptosis of HepG2 cells

After 12 days of induction and stimulation, the stem cells differentiated into mature $CD4^+CXCR5^+CD57^+$T cells (Fig. 3A). The concentration of IFN-γ in $CD4^+CXCR5^+CD57^+$T cells culture medium is significantly increased (Fig. 3B). The concentration of IL-6 in $CD4^+CXCR5^+CD57^+$T cells culture medium is significantly increased (Fig. 3C). The concentration of IL-21 in $CD4^+CXCR5^+CD57^+$T cells culture medium is significantly increased (Fig. 3D). The concentration of CXCL13 in $CD4^+$ $CXCR5^+$ $CD57^+$ T cell culture medium did not change (Fig. 3E).

The function of $CD4^+CXCR5^+CD57^+$T cells. Add 20% of the cell culture medium on the 12th day to the HepG2 cell culture medium. At 24 h and 48 h, apoptosis of HepG2 cells can be seen (Fig. 3F). Cells were detected by Annexin V-FITC/PI double staining method. The rate of apoptosis at 48 h was 6.04% (Fig. 3G). Observing the morphology of $CD4^+CXCR5^+CD57^+$T cells under a microscope, it can be seen that the cell size is small, the cytoplasm is small, and the nucleus is round and dense. Observing the killing effect on liver cancer, HepG2 cell apoptosis occurred at 48 h, and the number decreased, while at 72 h, all apoptosis occurred (Fig. 3F).

This experiment shows that stem cells can induce differentiation into $CD4^+CXCR5^+CD57^+$T cells and induce apoptosis of liver cancer cells by secreting IL-21 and IFN-γ.

HBV inhibits the number and function of $CD4^+CXCR5^+CD57^+$T cells, leading to chronic inflammatory reactions

HBV leads to a decrease in the number of $CD4^+CXCR5^+CD57^+$T cells. The number of $CD4^+CXCR5^+CD57^+$T cells in normal mouse accounted for 8.51 ± 1.58% of the total CD4 cells, while C57BL/6.Cg-Tg(HBV)Smoc mice was 2.16 ± 1.08%, indicating that HBV inhibits the number of $CD4^+CXCR5^+CD57^+$T cells, inhibit the body immune function (Fig. 4A). C57BL/6.Cg-Tg(HBV)Smoc mice serum levels of IL-6, IL-21, and IFN-γ decreased. (Fig. 4B, C, D). C57BL/6.Cg-Tg(HBV)Smoc mice serum CXCL13 concentration is decrease serum concentration (Fig. 4E). HBV detection of C57BL/6.Cg-Tg (HBV)Smoc mice showed that the DNA amount increased (Fig. 4F). The decrease in the number of $CD4^+CXCR5^+CD57^+$T cells leads to chronic inflammation. The liver and lung pathology of C57BL/6.Cg-Tg(HBV)Smoc mice showed local chronic inflammation (Fig. 4G).

Treg gene regulates $CD4^+CXCR5^+CD57^+$T cells

Detect the number of $CD4^+CXCR5^+CD57^+$T cells in $FOXP3^{-/-}$-C57BL/6J mice. The number of $CD4^+CXCR5^+CD57^+$T cells in

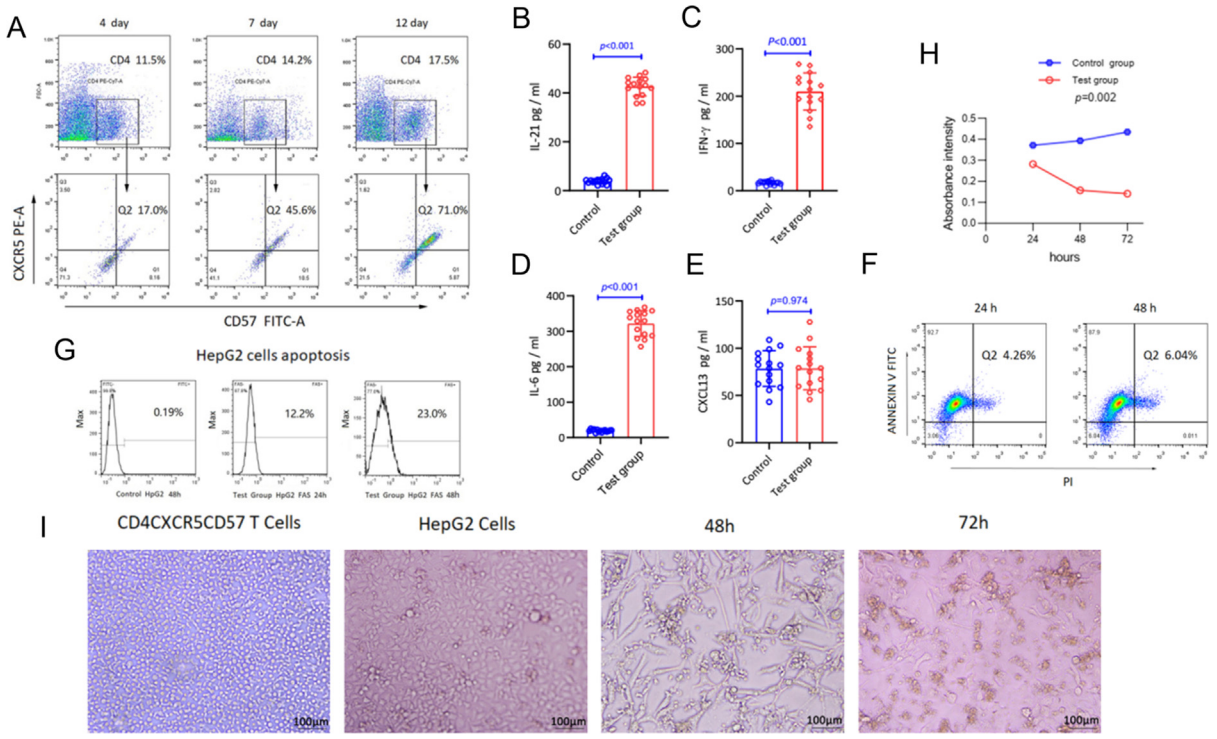

Fig. 3. IL-21 and IFN-γ induce stem cells to differentiate into $CD4^+CXCR5^+CD57^+$T cells. **(A)** After 12 days of induced differentiation, $CD4^+CXCR5^+CD57^+$T cells accounted for 68.1% ± 9.5% of $CD4^+$ T cells. **(B)** The concentration of IFN-γ secreted by $CD4^+CXCR5^+CD57^+$T cells was 210.0 ± 39.13 pg/ml, and the culture medium was 16.83 ± 3.53 pg/ml (n = 10, $P < 0.001$). **(C)** The concentration of IL-6 was 322.0 ± 36.2 pg/ml, the culture medium was 19.21 ± 3.55 pg/ml (n = 10, $P < 0.001$). **(D)** The concentration of IL-21 was 42.61 ± 3.84 pg/ml, the culture medium was 3.86 ± 1.11 pg/ml (n = 10, $P < 0.001$). **(E)** The concentration of CXCL13 was 78.76 ± 22.75 pg/ml, the culture medium was 78.52 ± 18.88 pg/ml (n = 10, $P = 0.974$). **(F)** $CD4^+CXCR5^+CD57^+$T cells induce the expression of CD95L in HepG2 cells, after 48 h, the positive expression rate of CD95L was 23.1 ± 3.7% (n = 10). **(G)** HepG2 cells apoptosis was detected by Annexin V-FITC/PI double staining method, after 48 h, the positive expression rate of apoptosis was 6.1 ± 2.6% (n = 10). **(H)** After 72 h of culture, $CD4^+CXCR5^+CD57^+$T cells inhibited HepG2 cells by 78.2% ($p = 0.002$ vs control group). **(I)** Observe the morphology of $CD4^+CXCR5^+CD57^+$T cells and HepG2 cells under a microscope, and the process of inducing HepG2 cells apoptosis.

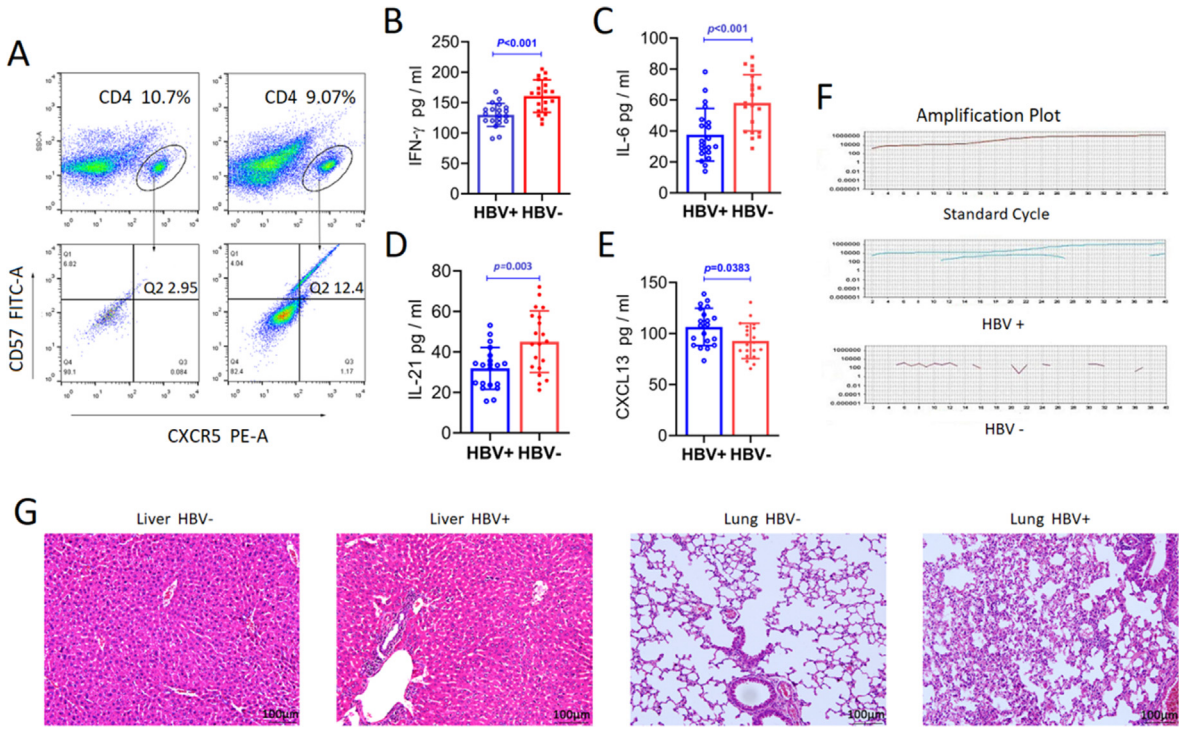

Fig. 4. HBV inhibits the number of $CD4^+CXCR5^+CD57^+$T cells and causes chronic inflammation. (**A**) $CD4^+CXCR5^+CD57^+$T cells in HBV-negative mice accounted for 8.51 ± 1.58% of the total number of CD4 cells, while HBV-positive was 2.16 ± 1.08%, (n = 10,vs HBV-negative mice group, $p < 0.001$). (**B**) The concentration of IL-21 in HBV positive serum was 31.88 ± 10.32 pg/ml, and HBV negative was 45.11 ± 15.21 pg/ml, (n = 10, vs HBV-positive mice group, $p < 0.001$). (**C**) The concentration of HBV positive IL-6 is 37.57 ± 17.0 pg/ml, and HBV negative is 58.17 ± 18.15 pg/ml, (n = 10, vs HBV-positive mice group, $p < 0.001$). (**D**) The concentration of HBV-positive IFN-γ is 129.7 ± 19.04 pg/ml, and HBV-negative is 160.8 ± 26.85 pg/ml, (n = 10,vs HBV-positive mice group, $p < 0.001$). (**E**) The concentration of CXCL13 in HBV-positive serum was 110.44 ± 37.14 pg/ml, and the concentration of HBV-negative serum was 92.73 ± 13.63 pg/ml, (n = 10,vs HBV-positive mice group, $p < 0.001$). (**F**) DNA testing for HBV mice. (**G**) The liver and lung pathology of HBV-positive mice were observed under a microscope, and local chronic inflammation was observed.

C57BL/6J mice is 10.51 ± 1.58%, and the Treg gene knockout increased significantly. The results show that the Treg gene can inhibit $CD4^+CXCR5^+CD57^+$T cells (Fig. 5A, B). Detect the serum cytokine concentration of $FOXP3^{-/-}$C57BL/6J mice. The serum IFN-γ concentration of $FOXP3^{-/-}$C57BL/6J mice increased significantly (Fig. 5C). The serum IL-21 concentration of $FOXP3^{-/-}$C57BL/6J mice increased significantly (Fig. 5D). The serum IL-6 concentration of $FOXP3^{-/-}$C57BL/6J mice increased significantly (Fig. 5E).

Pathological examination of liver and lung of $FOXP3^{-/-}$C57BL/6J mice. The results showed that when the Treg gene function was lost, the number of $CD4^+CXCR5^+CD57^+$T cells increased significantly, and a large number of inflammatory cell infiltrations were seen in the liver and lung. The liver was selected for CD4, CXCR5, and CD57 for IHC analysis and a large number of positive expressions were seen, while normal mice did not see infiltration (Fig. 5F).

IL-21 and IFN-γ induce $CD4^+CXCR5^+CD57^+$T cell formation to treat H22 liver cancer

Twenty C57BL/6J mice were divided into the treatment group and model group, each with 10 mice. 0.1 ml of H22 cells suspension was injected into the left chest at a concentration of 1×10^7/ml, and IL-21, IFN-γ was given an intraperitoneal injection, 1U/g, for 3 consecutive days after inoculation with H22 cells. The xenograft tumor was taken for analysis 14 days later.

Compared with the model group, H22 xenograft tumors in the IL-21 and IFN-γ treatment group were significantly reduced (Fig. 6A). The average weight of the xenograft tumors in the treatment group was 0.95 ± 0.24 g, while the model group was 2.10 g ± 1.14 g, comparison of two groups ($p < 0.001$) (Fig. 6B). The HE staining of the xenograft tumor showed that the tumor tissue grew in a nodular shape, and the tumor cells were abundant and crowded. Tumor cells vary in size and morphology, with large nuclei and extremely irregular nuclear membranes. There are large eosinophilic nucleoli in the nucleus, and necrotic tissue can be seen in large tumor foci (Fig. 6C). The number of $CD4^+CXCR5^+CD57^+$T cells in the treatment group was 19.17 ± 2.45%, and in the model group was 3.68 ± 0.78%, compared to treatment group ($p < 0.001$) (Fig. 6D).

Detect the concentration of serum cytokines. The serum IFN-γ concentration of mice in the treatment group was 202.7 ± 83.94 pg/ml, and the model group was 118.1 ± 76.85 pg/ml, comparing to treatment group ($p < 0.001$) (Fig. 6E). The serum IL-21 concentration in the treatment group was 38.59 ± 12.14 pg/ml, and the model group was 26.45 ± 5.90 pg/ml, compared of two group ($p = 0.035$) (Fig. 6F). The serum IL-6 concentration in the treatment group was 129.9 ± 34.7 pg/ml, and the model group was 224.6 ± 68.19 pg/ml, compared to treatment group ($p < 0.001$) (Fig. 6G). The serum CXCL13 concentration in the treatment group was 139.6 ± 40.9 pg/ml, and the model group was 78.2 ± 21.9 pg/ml, compared to treatment group ($p < 0.001$) (Fig. 6H). In the treatment group, the concentration of IL-21 and IFN-γ increased, and IL-6 decreased when the transplanted tumor was controlled; the concentration of CXCL13 increased, which increased the body immune response.

Detect the gene expression of IL-6, IL-21, CD57, and CXCR5 in the TME of xenograft tumors. The results showed that the gene expression of IL-21, CD57, and CXCR5 was significantly increased in the treatment group. Compared with the model group, IL-21 and CD57 ($p < 0.001$), and CXCR5 ($p < 0.01$). The gene expression of IL-6 in the treatment group decreased, compared with model group ($p < 0.001$) (Fig. 6I).

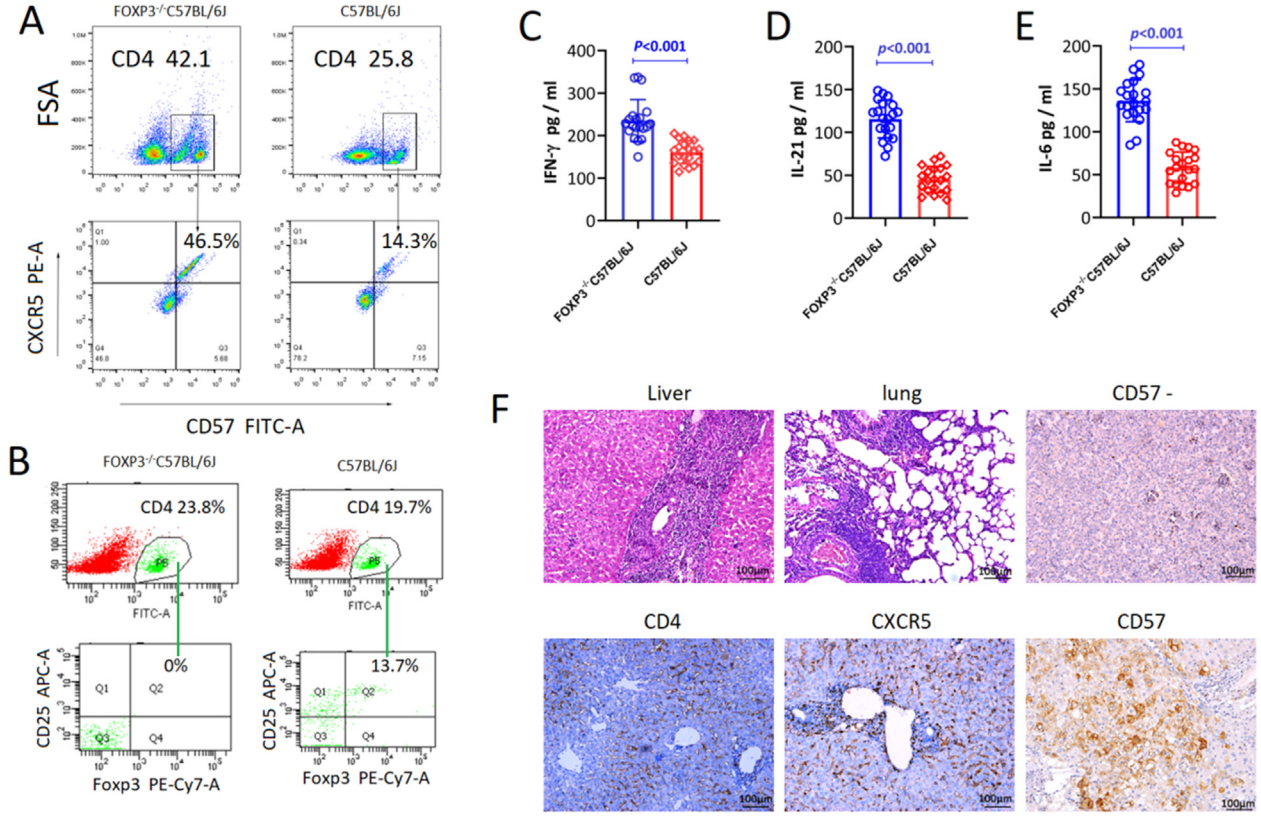

Fig. 5. Regulation of Treg gene on $CD4^+CXCR5^+CD57^+$T cells. (**A**) The number of $CD4^+CXCR5^+CD57^+$T cells in C57BL/6J mice is 10.51 ± 1.58%, the number of $CD4^+CXCR5^+CD57^+$T cells in Treg knockout mice was 34.68 ± 13.08%, which was significantly higher, (n = 10, vs C57BL/6J group, $p < 0.001$). (**B**) There is no expression of the number of $CD4^+CD25^+Foxp3^+$T cells in Treg knockout mice. (**C**) The serum IFN-γ concentration of the C57BL/6J group was 160.8 ± 26.85 pg/ml, and the serum IFN-γ concentration of $FOXP3^{-/-}$C57BL/6J mice was 235.7 ± 48.93 pg/ml, which increased significantly, (n = 10, vs C57BL/6J group, $p < 0.001$). (**D**) The serum IL-21 concentration of the normal group was 45.11 ± 15.21 pg/ml, $FOXP3^{-/-}$C57BL/6J was 115.5 ± 21.9 pg/ml, which increased significantly, (n = 10, vs C57BL/6J group, $p < 0.001$). (**E**) The serum IL-6 concentration of the normal group was 58.17 ± 18.15 pg/ml, and the serum IL-6 concentration of $FOXP3^{-/-}$C57BL/6J was 132.2 ± 24.4 pg/ml, which increased significantly, (n = 10, vs C57BL/6J group, $p < 0.001$). (**F**) The liver and lung pathology of $FOXP3^{-/-}$C57BL/6J mice showed a large amount of inflammatory cell infiltration, and IHC analysis showed a large number of positive expressions of CD4, CXCR5, and CD57.

Discussion

It has been found in clinical studies that IL-21 and IFN-γ secreted by $CD4^+CXCR5^+CD57^+$T cells have a killing effect on HCC. The number of $CD4^+CXCR5^+CD57^+$T cells in patients with liver cancer who survived for a long time increased significantly. At the same time, it was found that in patients with advanced liver cancer, the concentrations of IL-21 and IFN-γ decreased, while those with longer survival time increased. IL-21 participates in the process of Tfh cells assisting the proliferation and differentiation of B cells into plasma cells [17]. IL-21 is an immunoregulatory cytokine in HBV infection and plays an important role in the clearance of HBV [18]. In patients with chronic HBV infection, IFN-γ eliminates HBV by generating specific CD4 T cells [19]. Human umbilical cord mesenchymal stem cells (mesenchymal stem cells) transplantation combined with IFN-γ dual therapy can improve the clinical efficacy of patients with rheumatoid arthritis [20]. RNase L is an important part of the antiviral signal pathway mediated by interferon. It has shown anti-tumor effects in cancer treatment, RNase L can be used as a therapeutic target for lung cancer, and IFN-γ can be used as an auxiliary means to improve efficacy [21]. The combination of IFN-γ and IL-6 can enhance the molecular expression of MHC, inhibit TGF-β1, and gradually shrink tumors [22]. IL-21 can induce the production of IFN-γ and enhance the innate immune response related to NK cells [23].

This study shows that $CD4^+CXCR5^+CD57^+$T cells secrete IL-21 and IFN-γ. IL-21 induces B cell immunity. IFN-γ directly acts on tumor cells, and when combined with IL-6, it can enhance the molecular expression of MHC. It also acts on HBV in the TME and has the effect of killing liver cancer cells.

Stem cells can differentiate into $CD4^+CXCR5^+CD57^+$T cells after induction. At present, the treatment of liver cancer is still limited, and immunotherapy has made progress in the treatment of liver cancer. Based on the results of the CheckMate 040 study, the immune checkpoint inhibitor Nivolumab is approved for the treatment of sorafenib-resistant HCC patients in Asia. In the cohort study, the objective response rates of HBV, HCV, and those without infection were 13%, 14%, and 21%, respectively [24]. Pembrolizumab is used to treat sorafenib-resistant liver cancer, the total effective rate is 32%, the median progression-free survival is 4.5 months, and the median overall survival is 13 months [25]. The 12-month overall survival rate of atezolizumab-bevacizumab was 67.2%, and the median progression-free survival was 6.8 months, respectively. 329 patients receiving atezolizumab-bevacizumab of the 56.5%, grade 3 or 4 adverse events occurred [26]. Immunotherapy is still facing problems such as low remission rate, many toxic side effects, and short survival time. It is necessary to find new therapeutic targets. In this experiment, IL-21 and IFN-γ were induced to differentiate. $CD4^+CXCR5^+CD57^+$T cells accounted for 68.1 ± 9.5% of the total number of CD4 cells. They mainly secrete IL-21 and IFN-γ, which are effective in the treatment of liver cancer HepG2 cells. The experiment showed the effect of inducing its apoptosis. IL-21 and IFN-γ induce the formation of differentiated

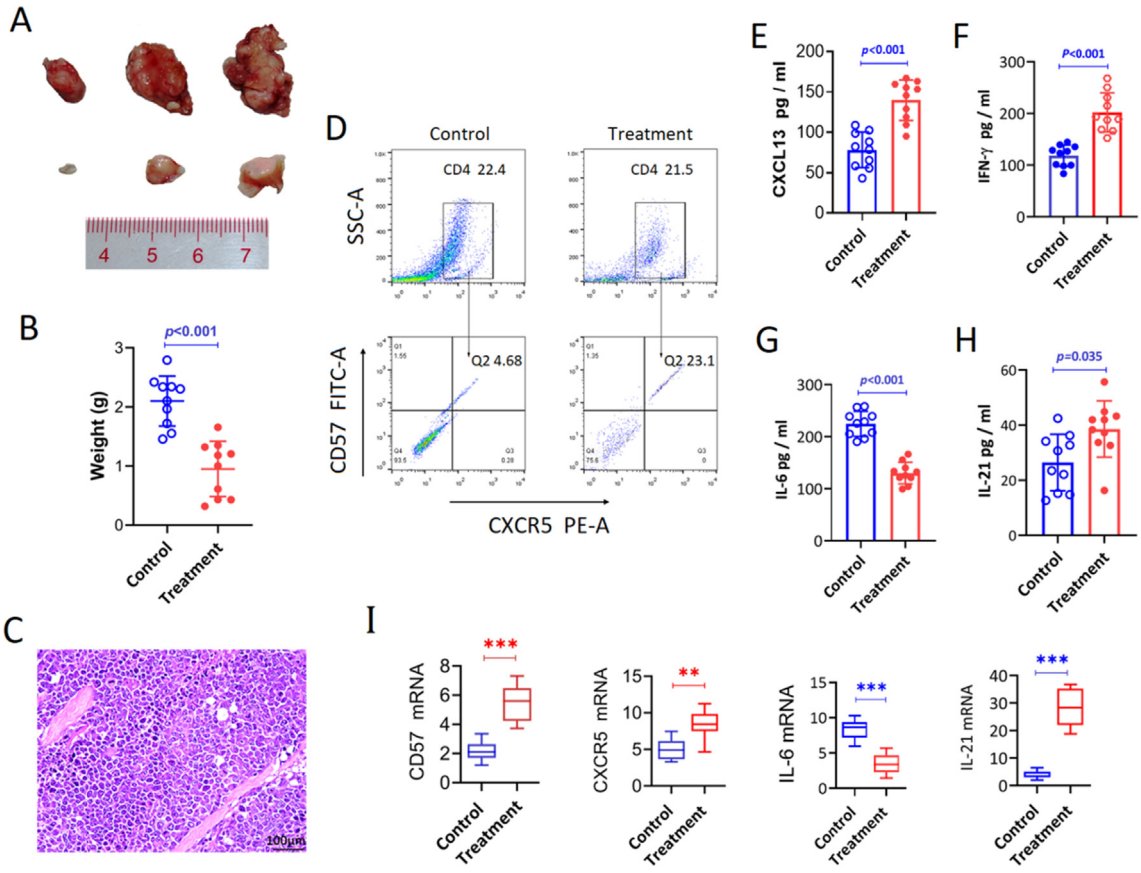

Fig. 6. IL-21 and IFN-γ induce $CD4^+CXCR5^+CD57^+$T cells to treat H22 liver cancer. (**A, B**) Compared with the model group, H22 xenograft tumor in the IL-21 and IFN-γ treatment group were significantly reduced. The average weight of the xenograft tumors in the treatment group was 0.95 g ± 0.24 g, while the model group was 2.10 g ± 1.14 g, (n = 10, vs treatment group, $p < 0.001$). (**C**) It is the pathological manifestation of an H22 xenograft tumor. (**D**) The number of $CD4^+CXCR5^+CD57^+$T cells in the treatment group was 19.17 ± 2.45%, and in the model group was 3.68 ± 0.78%, (n = 10, vs treatment group, $p < 0.001$). (**E**) The serum IFN-γ concentration of mice in the treatment group was 202.7 ± 83.94 pg/ml, and the model group was 118.1 ± 76.85 pg/ml, (n = 10, vs treatment group, $p < 0.001$). (**F**) The serum IL-21 concentration in the treatment group was 38.59 ± 12.14 pg/ml, and the model group was 26.45 ± 5.90 pg/ml, (n = 10, vs treatment group, $p = 0.035$). (**G**) The serum IL-6 concentration in the treatment group was 129.9 ± 34.7 pg/ml, and the model group was 224.6 ± 68.19 pg/ml, (n = 10, vs treatment group, $p < 0.001$). (**H**) The serum CXCL13 concentration in the treatment group was 139.6 ± 40.9 pg/ml, and the model group was 78.2 ± 21.9 pg/ml, (n = 10, vs treatment group, $p < 0.001$). (**I**) Gene expression of IL-6, IL-21, CD57, and CXCR5 in the microenvironment of xenograft tumor. *<0.05, **<0.01, ***<0.001, ns indicates normal.

$CD4^+CXCR5^+CD57^+$T cells, which may be a method for the treatment of liver cancer.

HBV infection inhibits the number and function of $CD4^+CXCR5^+$-$CD57^+$T cells, causing chronic inflammation. Chronic HBV infection affects approximately 375 million people worldwide. Current antiviral treatments can only effectively control, but cannot eliminate chronic HBV infection. HBV is still a high risk factor for liver cirrhosis and liver cancer, and the pathogenesis of HBV infection is poorly understood [27]. More evidences show that HBV interacts with hepatocyte innate immune signaling pathways and inhibits innate immunity. However, how HBV avoids the surveillance of innate hepatocyte immunity, and whether the re-triggering of innate hepatocyte immunity is effective for HBV, it is still unclear [28]. Intrahepatic gene expression profile showed that compared with the non-infected control group, the antiviral effector, interferon-stimulating gene, toll-like, and pathogen recognition receptor pathways of chronic hepatitis B patients were significantly down-regulated, and they were not directly related to HBV replication Sex [29]. In a liver environment without inflammatory infiltration, the adoptive transfer of engineered T cell therapy may be more effective and less risky. New T cell therapy is challenging to treat patients with chronic HBV, and the potential curative effect will further promote research in this field. Immunotherapy is a real treatment option for chronic HBV infection [30,31]. The expression of IL-2 and IL-21 in HCC patients was significantly lower than that in the control group. Supplementing recombinant human IL-2 and IL-21 in vitro expansion culture can enhance the cytotoxicity [32].

The number of $CD4^+CXCR5^+CD57^+$T cells in C57BL/6.Cg-Tg(HBV) Smoc was 2.16 ± 1.08%. Compared with normal mice, the number decreased significantly, indicating that HBV inhibits $CD4^+CXCR5^+$-$CD57^+$T cells. By detecting the decrease of serum IL-6, IL-21, and IFN-γ concentration in HBV transgenic mice. Through pathological examination and analysis, it can be seen that the liver and lungs have lymphocyte infiltration. This experiment shows that HBV infection inhibits the number and function of $CD4^+CXCR5^+CD57^+$T cells. If the number and function of the cells are increased, it may have a therapeutic effect on HBV.

$CD4^+CXCR5^+CD57^+$T cells are regulated by Treg genes. Treg cells suppress abnormal/excessive immune responses to self and non-self-antigens to maintain immune homeostasis. Infiltration of Treg cells into the TME occurs in a variety of mouse and human tumors [33,34]. Treg inhibition mechanism, Treg cells strip IL-2 from surrounding cells through its high-affinity IL-2 receptor, so that the responding T cells cannot obtain IL-2. CTLA-4 is also expressed in groups, and CTLA-4 down-regulates the expression of CD80/CD86 through antigen presenting cells (APCs), thereby depriving response T cells of costimulatory signals. Treg cells also produce immunosuppressive cytokines such as IL-10, which down-regulate APC function [35]. Treg cells easily penetrate into the TME, inhibit the anti-tumor immune response, and become an

obstacle to effective tumor immunotherapy. The development of new immunotherapies for a variety of inhibitory receptors, such as CTLA4, PD1, and LAG3, has been substantially expanded, but its efficacy still needs to be further improved [36].

Treg genes can inhibit $CD4^+CXCR5^+CD57^+$T cells. The serum IFN-γ, IL-2, and IL-6 concentrations of $FOXP3^{-/-}$C57BL/6J mice all increased. Treg plays a key role in suppressing spontaneous autoimmune diseases and promoting immune evasion of cancer. Some non-immune functions, such as tissue homeostasis and regeneration, are also attributed to Treg. New small molecules, biologics, and adoptive cell therapies are being tested for Treg responses [37]. This study shows that Treg cells can inhibit $CD4^+$-$CXCR5^+CD57^+$T cells in TME.

Therapeutic effect of $CD4^+CXCR5^+CD57^+$T cells in mouse H22 xenograft. Preclinical and clinical studies have shown that immune checkpoint treatment is beneficial to the survival of primary liver cancer. Anti-PD-1 antibody combined with local therapy or other molecular targeted drugs is an effective strategy for the treatment of HCC [38,39]. NK cells are enriched in liver hematopoietic cells, and have unique NK cell characteristics, protecting liver cells from hepatitis virus infection or cancer. In liver cancer, NK cells are damaged, so repairing the function of NK cells is a strategy for liver cancer [40]. The global burden of HCC is increasing and may soon exceed 1 million cases per year. Only 25% of liver cancers have potential targeting drivers. The median overall survival of new drugs such as first-line lenvatinib and second-line regorafenib, cabozantinib, and ramucirumab is still about 1 year, and breakthroughs in treatment are still needed [41].

The therapeutic effect of the combined application of IL-21 and IFN-γ on H22 xenograft tumor. Compared with the model group, the H22 xenograft tumor in the IL-21 and IFN-γ treatment groups were significantly reduced. HE staining showed that the tumor tissues grew in nodular shape, and the tumor cells were abundant and crowded. Tumor cells vary in size and shape, with large nuclei, extremely irregular nuclear membranes, and large eosinophilic nucleoli. The number of $CD4^+CXCR5^+CD57^+$T cells in the treatment group was significantly higher than that in the model group.

The concentration of serum IFN-γ and IL-21 in the treatment group increased significantly, the concentration of IL-6 decreased significantly, and the concentration of CXCL13 increased. In the treatment group, the expression of IL-21, CD57, and CXCR5 genes was significantly increased, and the expression of IL-6 genes was decreased. The combination of IFN-γ and IL-6 can enhance the molecular expression of MHC and enhance the therapeutic effect of $CD4^+CXCR5^+CD57^+$T cells.

Conclusions

The conclusion of the study is that the combination of IL-21 and IFN-γ induces stem cells to differentiate into $CD4^+CXCR5^+CD57^+$T cells, and Treg can control the increase in their number, while HBV can cause their number to decrease. $CD4^+CXCR5^+CD57^+$T cells can control the growth of liver cancer and prolong survival time.

Compliance with Ethics Requirements

All Institutional and National Guidelines for the care and use of animals (fisheries) were followed.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5). Informed consent was obtained from all patients for being included in the study.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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