Int J Clin Exp Med 2019;12(5):5333-5340
www.ijcem.com /ISSN:1940-5901/IJCEM0086434

Original Article

Exosomes derived from hepatocellular carcinoma inhibit CD4$^+$ and CD8$^+$ T cell function through the PD-1/PD-L1 pathway

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Received October 8, 2018; Accepted February 11, 2019; Epub May 15, 2019; Published May 30, 2019

Abstract: T cell function is significantly inhibited in hepatocellular carcinoma (HCC), although T cells play an important role in killing cancers. To illustrate the mechanisms of T cell inhibition in liver cancer, CD4$^+$ and CD8$^+$ T cell quantity and function were investigated in our research. First, we counted the numbers of CD4$^+$ and CD8$^+$ T cells in HCC peripheral blood by flow cytometry and found that CD4$^+$ and CD8$^+$ T cells were not obviously decreased compared to levels found in healthy volunteers. However, we found that the secretion of IFN-γ from CD4$^+$ and CD8$^+$ T cells, an important factor for T cell activation, was significantly decreased compared to the control group. Furthermore, we wondered whether CD4$^+$ and CD8$^+$ T cell suppression was mediated by hepatocellular carcinoma exosomes. Exosomes were successfully isolated from Huh-7 cell supernatant and verified through NanoSight analysis and transmission electron microscopy (TEM). CD4$^+$PD-1$^-$ and CD8$^+$PD-1$^-$ T cells were sorted from the peripheral blood of healthy volunteers by flow cytometry and cultured with RPMI-1640 medium. Then, CD4$^+$PD-1$^-$ and CD8$^+$PD-1$^-$ T cells were treated with HCC exosomes, and Western blot assays were used to discover whether levels of programmed cell death protein 1 (PD-1), a negative modulator of T cell response, were upregulated on these T cells. To examine how HCC exosomes are involved in CD4$^+$ and CD8$^+$ T cell inhibition, we researched whether PD-L1, the ligand for the PD-1 protein, was upregulated in HCC exosomes using a Western blot assay. Our experiments indicate that exosomes containing PD-L1 derived from hepatocellular carcinoma might induce PD-1 upregulation, resulting in CD4$^+$ and CD8$^+$ T cell suppression.

Keywords: Hepatocellular carcinoma, exosomes, CD4$^+$/CD8$^+$ T cells, PD-1, PD-L1

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers and the second leading cause of cancer-related deaths worldwide [1]. The limited means of early diagnosis results in advanced tumors in HCC, so the majority of patients in the advanced stage do not respond well to treatments [2]. Recent evidence on the tumor microenvironment has shown its crucial role in tumor progression and disease prognosis [3], and it has now become a major focus of research.

In the immune microenvironment, CD4$^+$ tumor-infiltrating T lymphocytes and cytotoxic CD8$^+$ T lymphocytes play positive roles in functional anti-tumor responses [4-6], and the quantity and function of T lymphocytes are the major contributors to inhibiting and killing tumor cells [7]. However, recent evidence suggests that intratumoral T cells display dysfunctional states and form various suppressive signals in the tumor microenvironment and fail to eliminate cancer cells.

An emerging area of research has shown that tumor-derived extracellular vesicles (EVs/exosomes) could modify the tumor microenvironment [8]. Exosomes are 30-150 nm nanoscale extracellular vesicles of endocytic origin that are released by most types of cells and circulate in bodily fluids. Exosomes carry specific components, including proteins, lipids, RNA, and DNA, and can function as cargo to transfer biological information as well as affect the physiological and pathological conditions of recipient cells [9].

Programmed cell death protein 1 (PD-1), a negative modulator of T lymphocytes, has an important role in T cell dysfunction [10]. PD-1 can also prevent the immune system from killing cancer cells [11, 12]. The PD-1 protein binds to its ligands, PD-L1 and PD-L2 [13, 14]. PD-L1, the ligand for PD-1, is highly expressed in several cancers, and the role of PD-1 in cancer immune evasion is therefore well established [15-17]. In our study, to observe $CD4^+$ and $CD8^+$ T cell immune function in hepatocellular carcinoma, we evaluated $CD4^+$ and $CD8^+$ T cell quantity and activation as well as PD-1 expression on these T cells. In addition, we further investigated the effects of exosomes derived from hepatocellular carcinoma on $CD4^+$ and $CD8^+$ T cell inhibition. Our research could provide new insight into hepatocellular carcinoma diagnosis and therapeutics.

Materials and methods

Patients

Peripheral blood samples were collected from early and middle stage patients with hepatocellular carcinoma at North China University of Science and Technology Affiliated Hospital between January and March, 2018. Healthy volunteers undergoing physical examinations in the hospital were taken as the concurrent control group. This research was approved by our Institutional Ethics Committee (Approval number: 18023). All patients signed a written informed consent.

Cells and antibodies

Huh-7 hepatocellular carcinoma cells were cultured with a complete DMEM medium at 5% CO2 in a 37°C atmosphere. $CD4^+PD\text{-}1^-$ and $CD8^+PD\text{-}1^-$ T lymphocytes were sorted from peripheral blood in the healthy control group and cultured in complete RPMI-1640 medium supplemented with anti-CD3, CD28 antibodies and IL-2. All antibodies were purchased from BD Biosciences, USA.

Flow cytometry assay

Human peripheral blood was collected from the HCC patients and from healthy volunteers. $CD4^+PD\text{-}1^-$ and $CD8^+PD\text{-}1^-$ T cells were sorted from the peripheral blood by flow cytometry using anti-human CD4, CD8, and PD-1 antibodies. Cells were measured by flow cytometric analysis (BD ARIA III BD Biosciences) to identify the $CD4^+$ and $CD8^+$ T cells and PD-1 expression on T cells.

qPCR

Total RNA was extracted from the T lymphocytes of the peripheral blood in liver cancer patients. The expression levels of IFN-γ were detected by quantitative real-time PCR using the SuperReal PreMix SYBR Green kit according to the instructions on the ABI 7500 real-time PCR system and the 7500 System Software-SDS 2.2 (Applied Biosystem). Each experiment was repeated three times, and similar results were obtained.

Exosome isolation and identification

The cell culture medium supernatant was collected from the HCC cells and cultured for 48-72 h, and the exosomes were isolated according to the kit protocols (GSTM Exosome Isolation Reagent, Guangzhou Geneseed Biotech Co., Ltd.), which depended on the extracellular vesicle PEG-based precipitation protocol. The exosomes were resuspended in 200 ul PBS and stored at -80°C for later use.

The exosome size distribution and concentration were analyzed by NanoSight technologies, and their characteristics were identified using a transmission electron microscope (TEM). The exosome pellet characteristics were examined and photographed with a transmission electron microscope (TEM; JEM-2100, Jeol, Japan). The isolation was verified by a nano-particle tracking analysis using a NanoSight N-300 (NanoSight Ltd, Amesbury, UK) to determine the concentration of EVs extracted.

Western blot assay

Equal amounts of protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred to a polyvinylidene fluoride membrane. Antibodies against PD-1, PD-L1, CD63 and GAPDH (BD Biosciences, USA) were incubated at 4°C overnight after blocking the nonspecific loci with 5% milk. After washing, the membranes were incubated with secondary antibody for 60 min and washed again. Proteins were detected using the ECL system

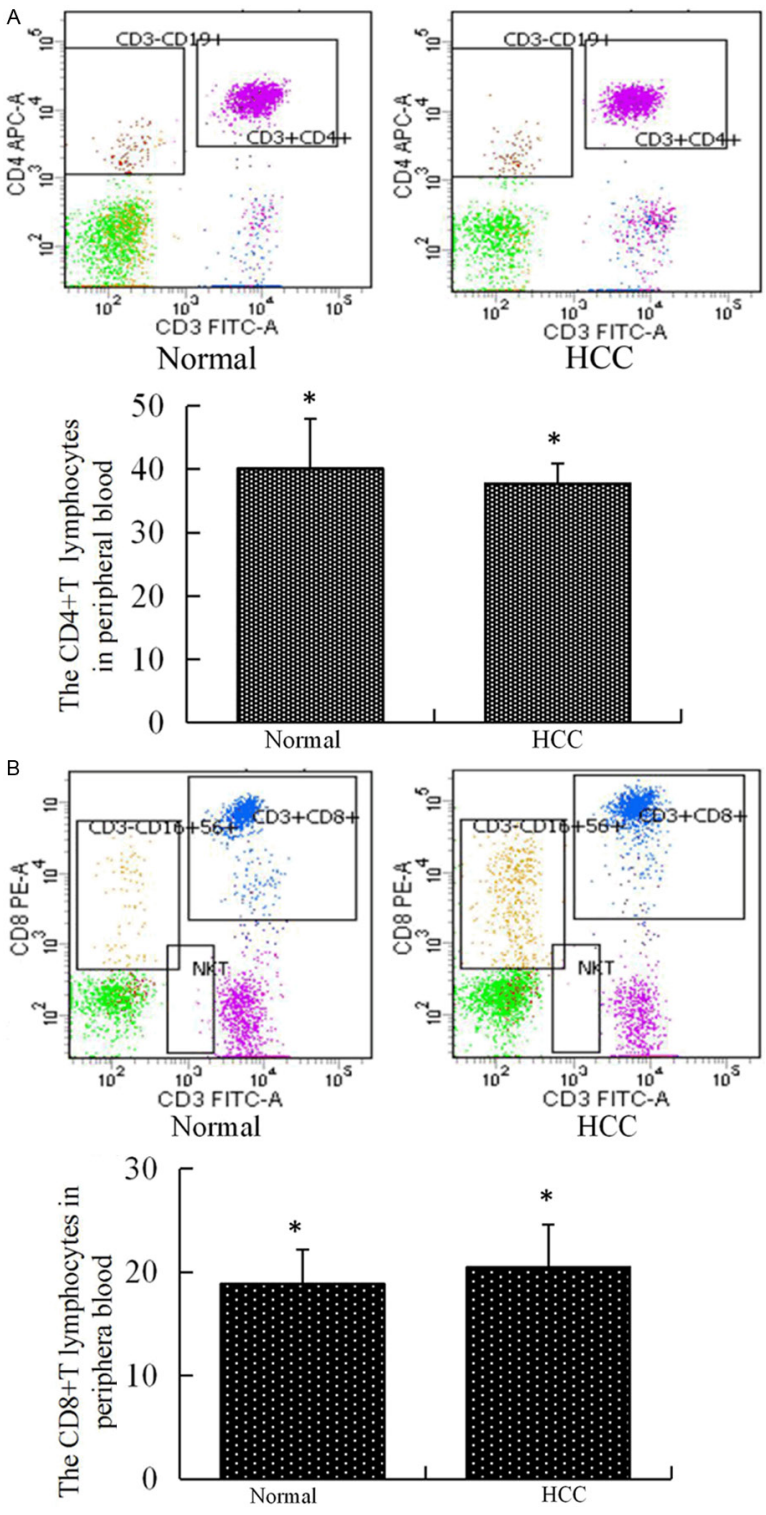

Figure 1. The distribution of CD4+ and CD8+ T lymphocytes in liver cancer patients by flow cytometry analysis. A. In peripheral blood of HCC patients, the quantity of CD4+ T cells was not decreased compared to the healthy volunteers group. *: P > 0.05. B. The quantity of CD8+ T lymphocytes in HCC peripheral blood was not obviously decreased compared with the normal group. *: P > 0.05.

(Pierce, Thermo, USA). Each experiment was repeated three times, and similar results were obtained.

Statistical analysis

The graphs were created using the Image Lab system (version 4.1; Bio-Rad Laboratories, Inc.). Student's *t*-test or one-way analysis of variance with Scheffe's F post hoc test were performed using SPSS 22.0 software (SPSS, Inc., Chicago, IL, USA). Data were presented as the means ± standard deviations. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

The distribution of CD4+ and CD8+ T cells in liver cancer patients

The quantity and function of CD4+ and CD8+ T cells exert important roles in anti-tumor immunity [7]. However, it has been shown that the immune state of HCC patients was significantly inhibited [18]. In our research, we investigated whether HCC immune suppression is related to the quantity and activation of CD4+ and CD8+ T cells. First, we speculated whether the immune dysfunction in HCC was caused by T cell quantity reduction. Therefore, the quantity of CD4+ and CD8+ T cells was evaluated through flow cytometry analysis. After collecting HCC patients' peripheral blood, we found that the number of CD4+ and CD8+ T cells were not decreased compared to those of the healthy volunteer group, as shown in **Figure 1A**, **1B**. This means that immune dysfunction has no relationship to the quantity

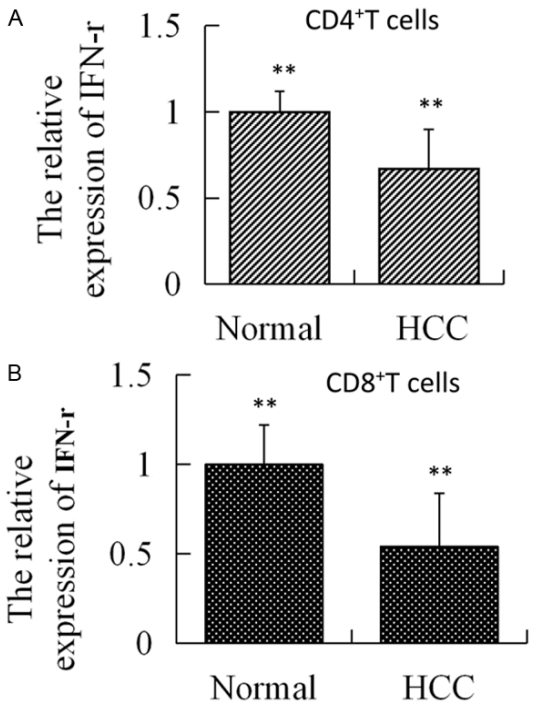

Figure 2. The function of T cells in hepatocellular carcinoma was detected by qPCR analysis. A. IFN-γ expression was decreased in the $CD4^+$ T cells of liver cancer patients' peripheral blood compared with the healthy control group. **: $P < 0.05$. B. The expression level of IFN-γ in $CD8^+$ T cells was obviously downregulated in HCC peripheral blood in comparison to the healthy control group. IFN-γ expression in $CD4^+$ or $CD8^+$ T cells was statistically significantly downregulated in liver cancer groups, **: $P < 0.05$.

of $CD4^+$ and $CD8^+$ T cells in the peripheral blood of hepatocellular carcinoma patients.

The function of $CD4^+$ and $CD8^+$ T cells was examined in hepatocellular carcinoma

As previous experiments described, the quantity of $CD4^+$ and $CD8^+$ T cells in HCC peripheral blood was not decreased in our research. We further speculated whether $CD4^+$ and $CD8^+$ T cell activation was prohibited, and was involved in HCC immune suppression. To evaluate the state of activation of $CD4^+$ and $CD8^+$ T cells, $CD4^+$ and $CD8^+$ T cells were sorted from HCC and healthy control group peripheral blood. We tested the levels of IFN-γ expression on these T cells, as IFN-γ is a potent cytokine in immune responses and an important indicator for T cell function [19-21]. qPCR analysis clearly indicated that IFN-γ expression was obviously decreased in comparison with the normal control group, as shown in **Figure 2**. This means that the activation of $CD4^+$ and $CD8^+$ T cells was significantly attenuated in HCC. The immune dysfunction in hepatocellular carcinoma was induced by $CD4^+$ and $CD8^+$ T cell suppression, but not by a reduced number of cells.

PD-1 overexpression induced T cell suppression in liver cancer patients

PD-1 is expressed on T cells and is involved in tumor immune suppression [22]. Therefore, we speculated that PD-1 overexpression might contribute to T-cell exhaustion in hepatocellular carcinoma. In our research, after collecting HCC peripheral blood, we detected the level of PD-1 expression on $CD4^+$ and $CD8^+$ T cells through flow cytometry analysis. The results indicated that PD-1 expression was increasingly upregulated on $CD4^+$ and $CD8^+$ T cells compared with the healthy control group, as shown in **Figure 3**. This suggests that PD-1 overexpression on $CD4^+$ and $CD8^+$ T cells plays a crucial role in HCC immune dysfunction.

Exosomes derived from hepatocellular carcinoma cells containing PD-L1 protein induced PD-1 expression

As studies show, tumor exosomes play a crucial role in modulating immune responses [23]. We speculated whether PD-1 upregulation on $CD4^+$ and $CD8^+$ T cells is mediated by exosomes released from hepatocellular carcinoma. In our study, exosomes released from Huh-7 cells were isolated using the Exosomes Isolation Kit, and their characteristics and concentration were verified by TEM and NanoSight technology, as shown in **Figures 4A** and **4B**. To examine the role of exosomes in modulating PD-1 expression on $CD4^+$ and $CD8^+$ T cells, $CD4^+PD\text{-}1^-$ and $CD8^+PD\text{-}1^-$ T lymphocytes from peripheral blood in the healthy control group were sorted by flow cytometry and cultivated for 5 days, as shown in **Figure 4C**. We tested PD-1 expression on $CD4^+PD\text{-}1^-$ and $CD8^+PD\text{-}1^-$ T cells after treatment with HCC exosomes. Western blot assays demonstrated that the PD-1 expression levels were obviously upregulated in both $CD4^+PD\text{-}1^-$ and $CD8^+PD\text{-}1^-$ T cells, as shown in **Figure 4D**. Therefore, exosomes derived from hepatocellular carcinoma could induce PD-1 expression, resulting in $CD4^+$ and $CD8^+$ T cell exhaustion in HCC.

PD-L1, the ligand for PD-1, is expressed in various tumors and plays a key role in tumor development [24, 25]. Because many molecules

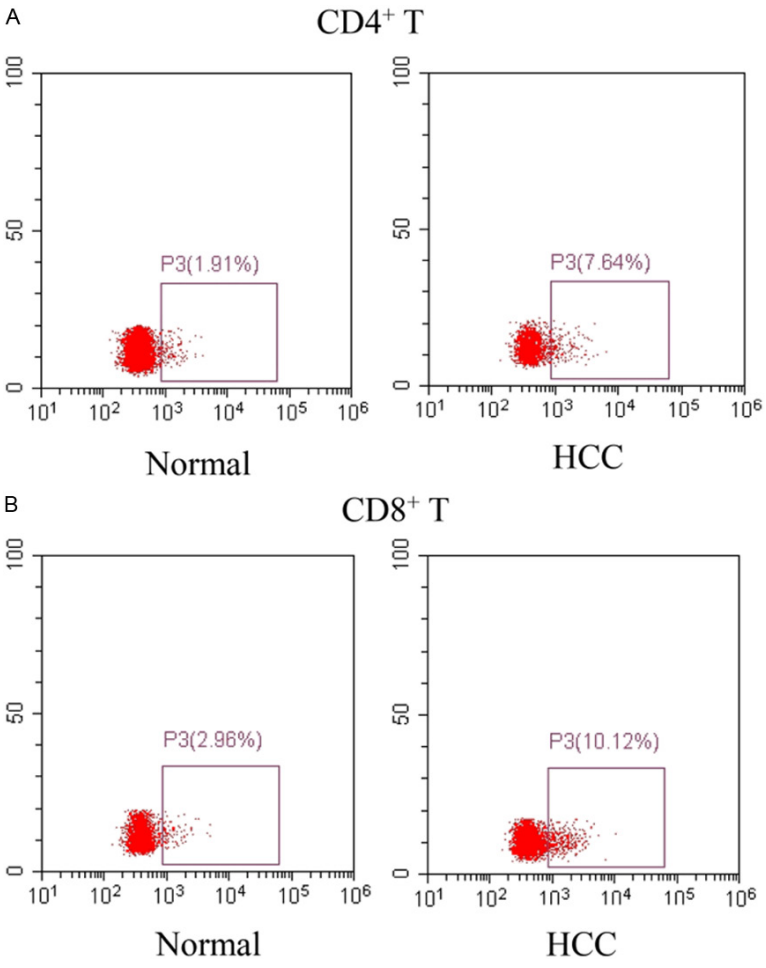

Figure 3. PD-1 expression on the T cells of liver cancer patients by flow cytometric analysis. A. Flow cytometry analysis showed that PD-1 expression was increased on $CD4^+$ T cells in HCC patients' peripheral blood compared with the normal control group. B. In HCC patients' peripheral blood, the level of PD-1 on $CD8^+$ T cells was obviously upregulated compared with the normal control group, as determined via flow cytometry analysis.

contained in exosomes could be transferred to recipient cells, we wondered whether exosomes from hepatocellular carcinoma include PD-L1 protein and affect the function of $CD4^+$ and $CD8^+$ T cells. Therefore, in our research, PD-L1 expression in HCC exosomes was detected by Western blot assay. Interestingly, we found that PD-L1 expression was upregulated in Huh-7 cells as well as in their exosomes through Western blot assay, as shown in **Figure 4E**. Thus, exosomes containing PD-L1 could affect $CD4^+$ and $CD8^+$ T cell function by upregulating PD-1 expression. Therefore, PD-L1 expression in tumor exosomes from hepatocellular carcinoma could induce PD-1 upregulation in $CD4^+$ and $CD8^+$ T cells and induce T cell dysfunction.

Discussion

Immune responses play key roles in tumor progression, and $CD4^+$ and $CD8^+$ T lymphocytes are among the major contributors to anti-tumor immunity [4-6]. Although the tissue of liver tumors was infiltrated with amounts of T lymphocytes, these T cells could not effectively eliminate cancer cells because of T cell dysfunction and exhaustion [26]. There are many studies related to T cell exhaustion in tumors. Reports have shown that Lnc-Tim3 is upregulated and negatively correlates with IFN-γ and IL-2 production in tumor-infiltrating $CD8^+$ T cells of HCC patients to influence tumor anti-immunity [27]. Research has also shown that HCCs with high proportions of PD-1^- high $CD8^+$ T cells expressing TIM3 and/or LAG3 and were more aggressive than HCCs with a smaller proportion of these cells [28]. In our research, we discovered that $CD4^+$ and $CD8^+$ T cells function was significantly impaired in hepatocellular carcinoma, while numerous $CD4^+$ and $CD8^+$ T cells infiltrated HCC peripheral blood.

PD-1 overexpression is one of the factors causing T cell exhaustion [3]. In multiple myeloma, higher levels of PD-1^+ $CD244^+$ or PD-1/Tim-$3^+CD57^+CD8^+$ T cells may be responsible for lower T-cell activation and T-cell immunodeficiency [29]. To investigate immune function in hepatocellular carcinoma, $CD4^+$ and $CD8^+$ T cell immune function and the levels of PD-1 expression in hepatocellular carcinoma were detected in our research. We tested the effect of PD-1 overexpression on $CD4^+$ and $CD8^+$ T cells and further investigated the mechanisms of this phenomenon.

Tumor-derived exosomes (TEX) are harbingers of tumor-induced immune suppression that carry immunosuppressive molecules and fac-

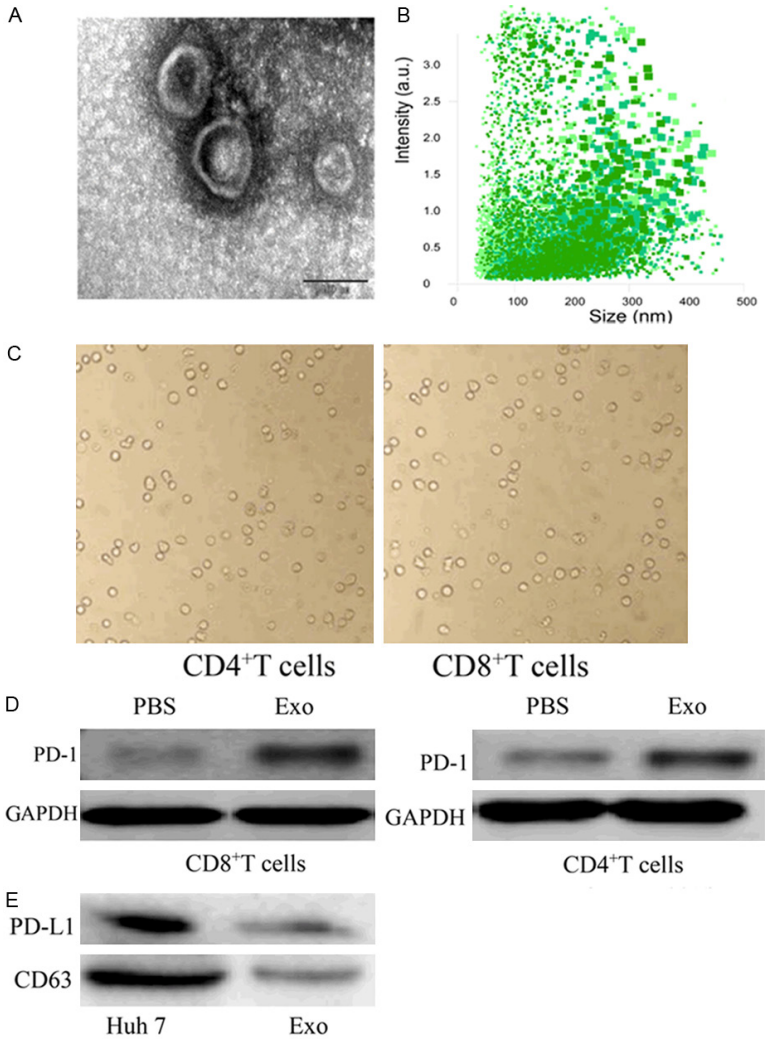

Figure 4. Hepatocellular carcinoma exosomes containing PD-L1 induced PD-1 expression on T lymphocytes. A. The characteristics of the exosomes released from the Huh-7 cells were identified by transmission electron microscopy; the exosomes had round vesicles and a proportional size. B. Exosome concentration was analyzed by NanoSight tracing technology. C. After sorting from the normal volunteers' peripheral blood using flow cytometry, the $CD4^+PD\text{-}1^-$ and $CD8^+PD\text{-}1^-$ T cells were cultured in a RPMI-1640 complete medium. D. Western blot assay indicates that the presence of PD-1 on T cells was induced after treatment with HCC exosomes. E. The levels of PD-L1 expression were detected in both Huh-7 cells and their exosomes through Western blot assay.

tors such as proteins, genomic DNA, mRNA, microRNAs, and tumor-associated antigens to immune cells, thereby downregulating antitumor immunity to promote tumor progression and the pathophysiological process [30]. Exosomes can also carry ncRNAs to local and distant cell populations; thyroid cancer stem-like cell exosomes transfer lncRNAs, importantly linc-ROR, to induce epithelial-mesenchymal transition (EMT) and inculcate the local tumor microenvironment and the distant metastatic niche [31]. Exosomes released from glioblastoma (GBM)-derived stem cells (GSCs) can induce an immunosuppressive microenvironment, and exosomes functionally mediate the immune suppressive switch because they contain the components of the transducer and activator of the transcription 3 (STAT3) pathway, revealing upregulation of PD-L1 in GSC exosome-treated monocytes [32]. Exosomes isolated from the plasma of patients with head and neck squamous cell carcinoma (HNSCC) carry PD-L1 and suppress T-cell functions [33]. Therefore, the function of exosomes from hepatocellular carcinoma on $CD4^+$ and $CD8^+$ T cell exhaustion were discussed in our research. We tested PD-1 upregulation on $CD4^+$ and $CD8^+$ T cells when treated with exosomes released from hepatocellular carcinoma. The expression of PD-L1 on tumor cells could inhibit anti-tumor responses through the engagement of PD-1 on effector T cells [34, 35]. In our research, we detected PD-L1 expression on exosomes derived from hepatocellular carcinoma cells using Western blot assays. Therefore, HCC exosomes containing PD-L1 in our research were demonstrated to play an important role in $CD4^+$ and $CD8^+$ T cell immunosuppression.

In summary, this research investigated the immune state of $CD4^+$ and $CD8^+$ T cells in hepatocellular carcinoma peripheral blood and further demonstrated that hepatocellular carcinoma exosomes containing PD-L1 play a crucial role in HCC immune dysfunction, thereby providing novel insights into diagnosis using th-

em as biomarkers, as well as suggesting an approach for tumor therapeutics.

Acknowledgements

This study was supported by the Natural Science Foundation of Hebei Province, China (Grant no. H2016209007), the Project for Scientific Research of the Health and Family Planning Commission of Hebei (grant no. 20180754), the State Administration of Traditional Chinese Medicine of Hebei (grant no. 2018182), the Colleges and Universities in Hebei Province Science and Technology Research Project (ZD2018090) and the Innovation Project for Postgraduates of North China University of Science and Technology, Hebei Province, China (grant no. 2018S40). The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Disclosure of conflict of interest

None.

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