SALL4-mediated upregulation of exosomal miR-146a-5p drives T-cell exhaustion by M2 tumor-associated macrophages in HCC

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ABSTRACT
Emerging evidence indicates that cancer cell-derived exosomes contribute to cancer progression through the modulation of tumor microenvironment, but the underlying mechanisms are not fully elucidated. Here, we reported that hepatocellular carcinoma (HCC)-derived exosomes could remodel macrophages by activating NF-κB signaling and inducing pro-inflammatory factors, and resulted in M2-polarized tumor-associated macrophages. In addition, the expression of IFN-γ and TNF-α was inhibited, while the expression of inhibitory receptors such as PD-1 and CTLA-4 was upregulated in T cells by HCC-derived exosome educated macrophages. Data also revealed that HCC exosomes were enriched with miR-146a-5p and promoted M2-polarization. Further investigation demonstrated that the transcription factor Sal-like protein-4 (SALL4) was critical for regulating miR-146a-5p in HCC exosomes and M2-polarization. Mechanistically, SALL4 could bind to the promoter of miR-146a-5p, and directly controlled miR-146a-5p expression in exosomes. Blocking the SALL4/miR-146a-5p interaction in HCC reduced the expression of inhibitory receptors on T cells, reversed T cell exhaustion, and delayed HCC progression in DEN/CCL-induced HCC mice. In conclusion, identification of a role of the exosomal SALL4/miR-146a-5p regulatory axis in M2-polarization as well as HCC progression provides potential targets for therapeutic and diagnostic applications in liver cancer.

Introduction
Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death in Asia and the fifth most common solid tumor worldwide. It attracts significant attention due to the aggressive nature, high mortality and low response rate to treatments. Immune response involving the interplay among macrophages, monocytes, dendritic cells (DCs), neutrophils, and lymphocytes is a physiological response for tissue regeneration, particularly hepatic repair after tissue injury or infection. However, in tumor microenvironment, anti-tumor immune response can be switched to a pro-tumor response. During tumor progression, T cells are prone to apoptosis, and T cell-mediated anti-tumorigenic response is markedly impaired, characterized by low secretion of IL-2, TNF-α, and IFN-γ and a high expression of inhibitory receptors such as PD-1. Clinical data show that PD-1/PD-L1 expression is positively associated with angiogenesis, tumor size, and tumor stage classification in patients with HCC. Thus, therapeutic strategy based on improving tumor microenvironment in HCC is gaining attention.

Macrophages, as innate immune cells, perform phagocytic clearance of pathogens and apoptotic cells, and as antigen presenting cells (APC), present antigen to adaptive immune cells. Macrophages, as dominant immune-related stromal cells in the tumor microenvironment, are crucial to tumor progression. Macrophages display diverse phenotypes, including classically activated (M1) or alternatively activated (M2). M1 macrophages that differentiated in response to IFN-γ stimulation are involved in Th1-type responses and characterized by elevated expression of major histocompatibility complex (MHC) class II, generation of nitric oxide (NO) and reactive oxygen species, as well as the ability to kill pathogens and infected cells. M1 macrophages promote abnormal cell apoptosis by producing pro-inflammatory cytokines such as IL-12 and TNF-α, resulting in the inflammation and the exacerbated tissue damage. In contrast, M2 macrophages are generated under the stimulation of IL-4 or IL-13 and involved in Th2-type responses. M2 macrophages are characterized by high levels of scavenging molecules such as mannose receptor CD206 that functions in endocytosis and phagocytosis of the perivascular tumor microenvironment, and scavenger receptor CD163 (recruiting Th2 cells) and cytokines such as IL-10. Meanwhile, M2 macrophages express the increased level of arginase, an enzyme for the synthesis of collagen, and thereby directly promotes cell division and tissue repair. Imbalance of M1 and M2 polarization is often implicated in driving pathophysiological complications. Tumor-associated macrophages (TAMs) are generally considered to be more closely resembling M2 phenotypes. Especially, tumor-infiltrating macrophages are more M2 tumor-promoting macrophages but not M1 antitumor macrophages. Delineating the processes that
underlie the phenotypic transition of macrophages will provide a novel and insightful understanding of tumor progression.

Exosomes are extracellular vesicles (30–150 nm) originating from the multivesicular body, and can be isolated from diverse body fluids and cell culture supernatants.\textsuperscript{18} They contain various types of molecules, including proteins, RNAs, DNAs, and lipids.\textsuperscript{19,20} A series of studies have identified exosomes as one of the important mediators of the interaction between tumor and surrounding non-tumor cells during cancer progression and metastasis. On the one hand, exosomes can directly modulate tumor cell function. Zakaria Y et al. revealed that tumor-derived exosomes delivered oncogenic nucleic acids and proteins to modulate the activity of normal cells, and accelerate tumorigenesis, metastasis in prostate cancer, breast cancer, and B-cell lymphoma.\textsuperscript{21–23} On the other hand, exosomes also can indirectly promote tumorigenesis by educating immune cells of the microenvironment, by suppressing T cell activation and natural killer (NK) cell-mediated antitumor activity, or other unknown mechanisms.\textsuperscript{19} Previous studies have reported that cancer cell-derived miR-1246, miR-21 and miR-222-3p can be delivered into macrophages via exosomes, educating macrophages toward tumor supportive and anti-inflammatory state, to reprogram the tumor microenvironment.\textsuperscript{6,22–25} We speculated that HCC cell-derived exosomes could remodel the tumor microenvironment by immunosuppression, and facilitate HCC disease progression. However, the potential underlying mechanisms are currently unknown.

In this study, the exosome-educated macrophages were generated by the treatment of HCC-derived exosomes.\textsuperscript{26} We discovered a mechanism for M2-polarization by exosome-based communication between tumor and macrophages residing in the microenvironment, which form a distinct subpopulation of tumor supportive macrophages. Our findings identified that SALL4, a zinc finger transcription factor that considered as inseparable with malignant proliferation of tumor cells,\textsuperscript{27} was enriched in tumor tissue, and mediated regulation of miR-146a-5p in HCC-derived exosomes can be potentially exploited for therapeutic and diagnostic applications in liver cancer.

**Results**

**HCC-derived exosomes activated macrophages**

The function of T cells was impaired in HCC due to the increased expression of inhibitory receptors and decreased secretion of inflammatory cytokines.\textsuperscript{28} In this study, HCC mouse model was established after DEN/CCL\textsubscript{4} administration for 40 weeks (Figure S1(a,b)). Characterization of the model exhibited up-regulation of inhibitory receptors, including Tim-3, LAG-3, CTLA4, TIGIT, and PD-1, on liver T cells (Figure S1(c)). The direct influence of DEN/CCL\textsubscript{4} on T-cell exhaustion was excluded by treatment with DEN/CCL\textsubscript{4} in vitro (Figure S1(d)).

To identify whether exosomes derived from HCC contributed to the formation of tumor microenvironment and tumor progression, exosomes were isolated from the supernatant of Hepa1-6 cells by ultracentrifugation. Exosomes were verified as small vesicles of approximately 100 nm in size by transmission electron microscopy (TEM), and with the expression of CD63 and CD81 (Figure 1(a)). The size distribution of the exosomes was predominantly within the range of 50–150 nm (Figure 1(b)).

Tumor-associated macrophages (TAMs), which phagocytose multiple cell fragments or proteins, trigger tumorigenic signals to enhance cancer cell growth, invasion, and metastasis during HCC progression.\textsuperscript{29} TAMs expressed high levels of dysfunctional molecules, which interacted with T cells and mediated T cell exhaustion in HCC.\textsuperscript{30–32} Therefore, firstly, we determined whether HCC-derived exosomes were involved in the activation of macrophages. The HCC-derived exosomes were labeled with DiD dye and incubated with peritoneal macrophages (PMs) from healthy mice. Confocal high content analysis showed that the DiD-labeled exosomes were efficiently internalized by PMs within 30 min of incubation (Figure 1(c)). Moreover, exposure to exosomes activated NF-κB in PMs in a dose-dependent manner, accompanied by the increase in pro-IL-1β, tnf-α, and il-1β (Figure 1(d,e)). Similar results were seen in RAW264.7 cells (Figure S2(a)). The dose 50 μg/mL was used in the following in vitro experiments unless otherwise noted. These data indicate that HCC-derived exosomes could be taken up by macrophages resulting in their activation.

**HCC-derived exosomes promoted M2 polarization**

TAMs exhibit M2 phenotype and promote tumor development.\textsuperscript{31} To determine whether HCC-derived exosomes regulated macrophage polarization, the populations of M1 and M2 macrophages in PMs incubated with exosomes were analyzed by Flow cytometry. We observed that the proportion of CD11b\textsuperscript{+}F4/80\textsuperscript{+}CD206\textsuperscript{+} macrophages was significantly increased following exposure to exosomes from Hepa1-6 cells (Figure 2(a) left) or serum of DEN/CCL\textsubscript{4}-treated mice in vitro (Figure 2(a) right). Furthermore, mRNA levels of M2 phenotype markers including ccl17, ccl22 and arg-1, as well as the production of ARG-1 in the supernatant were upregulated in PMs treated with HCC-exosomes (Figure 2(b)).

Studies have shown that the activation of STAT3 signaling pathway mediated the M2-type polarization of macrophages,\textsuperscript{32,33} while STAT1 signaling pathway mediated the polarization of M1-type macrophages.\textsuperscript{33,34} Here, we observed that STAT3 phosphorylation was elevated in PMs incubated with HCC-exosomes (Figure 2(c)), while the phosphorylation of STAT1 decreased (Figure 2(d)) accompanied by the reduction of ROS production (Figure 2(e)). Similar results were observed in RAW264.7 and human THP-1 cells (Figure S2(b–d)). These results indicated that HCC-derived exosomes promoted polarization of macrophages toward M2 tumor-associated macrophages.

**Macrophages educated with HCC-derived exosomes inhibited T cell response**

While analyzing the phenotype of HCC-exosome “educated” macrophages, we noticed that the proportion of CD11b\textsuperscript{+}F4/80\textsuperscript{+}CD169\textsuperscript{+} macrophages, which was associated with suppressive activity of macrophages and immune tolerance,\textsuperscript{35,36} was significantly upregulated in the PMs treated by HCC-exosomes compared with the control group (Figure 3(a)).
However, the antigen presenting MHC-II was downregulated by HCC-exosome treatment, while the ligands related to macrophage dysfunction such as PD-L1 and CD80 were upregulated (Figure 3(b)). These phenomena were also observed in RAW264.7 cells (Figure S3). Subsequently, we determined whether HCC-exosome “educated” macrophages could affect the function of T cells. We observed that the inhibitory receptors PD-1, TIGIT, and CTLA4 on CD3+ T cells were increased by co-culturing with PMs treated with HCC-exosomes compared with T cells co-cultured with untreated PMs (Figure 3(c)). In addition, the production of IL-2, IFN-γ, and TNF-α in CD3+ T cells was lowered when co-cultured with HCC-exosome “educated” PMs (Figure 3(d)). No significant difference was observed between HCC-exosome treated and untreated T-cells. Similarly, PMs treated by the exosomes from serum of DEN/CCL4-treated mice upregulated PD-1 on T cells (Figure S4(a)). Human THP-1 treated by exosomes from HepG2 or H7402 cell lines downregulated the inducible co-stimulator ICOS and Granzym B on T cells (Figure S4(b)).

To further confirm the function of HCC-exosome “educated” macrophages in vivo, CD11b+F4/80+ macrophages derived from bone marrow (BMDMs) were prepared (Figure S5(a,b)) and treated with HCC-exosomes in vitro. As shown in Figure 3(e), the proportion of CD206+ cells increased, while the iNOS+ cells decreased in the presence of HCC-exosomes, indicating M2 polarization. C57BL/6 mice were administrated with 200 μL of clodronate liposomes by intravenous injection to deplete macrophages (Figure S5(c)), and then transferred with control or HCC-exosome treated BMDMs. Compared with the hepatic T cells from mice transferred with control BMDMs, the inhibitory receptors Tim-3 and TIGIT were upregulated on T cells from mice transferred with HCC-exosome treated BMDMs, while the production of IL-2 and TNF-α was decreased (Figure 3(f)). Meanwhile, the cytolysis activity of T cells against HCC cells (Figure 3(g)) and the expansion ability of T cells (Figure 3(h)) were suppressed by transferring HCC-exosome treated BMDMs. Thus, HCC-exosome “educated” macrophages displayed immunosuppressive activity by inducing T cell exhaustion.

**MiR-146a-5p in HCC-derived exosomes exerted key role on macrophage M2-polarization**

MicroRNAs (miRNAs) are selectively packaged into exosomes, which largely dictate the effects of exosomes on recipient cells. Therefore, several miRNAs involved in immunoregulation were analyzed in exosomes derived from Hepa1-6 cells, including miR-146a-5p, miR-155, miR-26a, and let-7f, which were either up-regulated or down-regulated in HCC and closely related to macrophage-polarization. Intriguingly, miR-146a-5p was the most abundant one among the four miRNAs expressed in Hepa1-6, H22, HepG2 and H7402 exosomes (approximately 100-fold higher relative to U6) (Figures 4(a) and S6(a)). And, its expression was remarkably higher in Hepa1-6 and H22 exosomes than healthy mouse hepatocytes (Figures 4(b) and S6(b)). However, the transfection of miR-146a-5p inhibitors could inhibit CD206+ macrophages significantly (Figures 4(c) and S6(c)) and down-regulated p-STAT3 and C/EBPβ (Figure 4(d)) in RAW264.7
cells and PMs incubated with HCC-exosomes, which suggested that miR-146a-5p in HCC-exosomes is an important mediator of M2 polarization.

To test this hypothesis, miR-146a-5p was overexpressed in exosomes by miR-146a-5p overexpressing vector (ov-146a) in Hepa1-6 (Figure S6(d)). We found the overexpression of miR-146a-5p in Hepa1-6 cells significantly increased the level of miR-146a-5p in the secreted exosomes (Figure S6(e)), which declined the expression of \( \text{tnf-} \alpha \) (Figure 4(e)) and promoted the differentiation of CD206\(^+\) macrophages (Figures 4(f) and S6(f)). In contrast, miR-146a-5p inhibiting vector (inh-146a) decreased the expression of miR-146a-5p in the Hepa1-6-derived exosomes, which up-regulated the level of \( \text{tnf-} \alpha \) and suppressed the generation of CD206\(^+\) macrophages significantly. Meanwhile, compared with exosomes from blank mice, exosomes from DEN/CCL\(_4\)-treated mice could significantly increase the percentage of CD206\(^+\) macrophages, while exosomes from sh-SALL4-tranduced mice did not show significant influence (Figure 5(d)). Consistently, the ratio of CD206\(^+\) RAW264.7 cells was significantly lowered by co-incubation with exosomes derived from sh-SALL4-treated Hepa1-6 cells compared with exosomes derived from sh-control-treated Hepa1-6 cells (Figure 5(e)), accompanied by downregulation of C/EBP\(\beta\) and p-STAT3 (Figure 5(f)).

Finally, we tried to clarify if SALL4 could directly regulate the expression of miR-146a-5p in HCC. We observed that silencing SALL4 decreased miR-146a-5p significantly in HCC cells (Figure S8) and the derived exosomes (Figure 5(g)) in vitro. And, ChIP analysis clearly demonstrated that SALL4 could

Transcription factor SALL4 controlled miR-146a-5p expression in HCC-derived exosomes

SALL4, a zinc finger transcription factor, was recently demonstrated to be highly expressed in HCC, and regulated by STAT3 transcription factors.\(^{27}\) We found SALL4 expression was increased during HCC development accompanied by the increasing number of M2-like macrophages, while silencing SALL4 via hydrodynamic injection of sh-SALL4 vector in DEN/CCL\(_4\)-induced HCC mice (Figure S7) significantly decreased the number of M2-like macrophages (Figure 5(a,b)). So, we speculated that miR-146a-5p was regulated by SALL4.

To verify whether SALL4 was involved in exosome-induced M2 polarization by regulating miR-146a-5p, we analyzed the level of miR-146-5p in exosomes from the serum of HCC mice. The results showed that the level of miR-146-5p in the exosomes was elevated by DEN/CCL\(_4\) treatment, and prevented by sh-SALL4 (Figure 5(c)). Meanwhile, compared with exosomes from blank mice, exosomes from DEN/CCL\(_4\)-treated mice could significantly increase the percentage of CD206\(^+\) macrophages, while exosomes from sh-SALL4-tranduced mice did not show significant influence (Figure 5(d)). Consistently, the ratio of CD206\(^+\) RAW264.7 cells was significantly lowered by co-incubation with exosomes derived from sh-SALL4-treated Hepa1-6 cells compared with exosomes derived from sh-control-treated Hepa1-6 cells (Figure 5(e)), accompanied by downregulation of C/EBP\(\beta\) and p-STAT3 (Figure 5(f)).

Figure 2. HCC-derived exosomes promoted macrophages toward M2-polarized tumor-associated macrophages.

PMs incubated with exosomes from Hepa1-6 cells (left) or the serum of DEN/CCL\(_4\)-induced HCC mice (right) for 24 h. CD206 expression was analyzed by Flow cytometry (a). Gene expression of ccl22, ccl17, and arg-1 was assayed by qPCR and ELISA (b). The activation of STAT3 in PMs was analyzed by western blot (c). The activation of STAT1 in PMs was analyzed by Flow cytometry (d). Representative micrographs of immunofluorescence analysis of ROS in PMs (e). Exo, exosomes. Data were representative of three independent experiments, statistical significance was determined as ***p < 0.001, **p < 0.01 and *p < 0.05 compared with control.
bind to the miR-146a-5p promoter (Figure 5(h)). In addition, we found the transcription activity of miR-146a-5p promoter was enhanced by overexpressing of SALL4 but repressed by silencing of SALL4 (Figure 5(i)). These data indicate that direct binding of SALL4 to the miR-146a-5p promoter regulated M2 polarization.

**SALL4 could switch the dysfunction of T cells and promote tumor development in DEN/CCL4-induced HCC**

To verify the key role of SALL4 in T cell exhaustion, we analyzed the changes of inhibitory receptors in DEN/CCL4-induced HCC mice treated by sh-SALL4. The results showed that the administration of sh-SALL4 significantly lowered the inhibitory receptors TIGIT, LAG-3 and PD-1 on T cells of DEN/CCL4-treated HCC mice (Figure 6(a)), but enhanced the production of IL-2 and TNF-α as well as the activatory receptor NKG2D (Figure 6(b)). Additionally, silencing SALL4 could obviously reduce the expression of afp, ki67 and pcna in hepatocytes (Figure 6(c)) and the tumor diameter (Figure 6(d,e)), accompanying with low degree of malignancy after administration for 30 weeks (Figure 6(f)). These results confirm that SALL4 activation enhances the expression of inhibitory receptors in T cells and promotes T cell exhaustion, accelerating HCC progression.
In the present study, we found that HCC cell-secreted miR-146a-5p could be delivered by exosomes into macrophages, switch the cytokine profile and attenuate antigen presentation of macrophages, and promote macrophages toward M2-polarized tumor-associated macrophages. Significantly, macrophages educated with HCC-derived exosomes impaired T cell functions. Furthermore, we found SALL4 could bind to the promoter and promote the expression of miR-146a-5p, while silencing SALL4 inhibited the expression of inhibitory receptors and reversed T cell exhaustion. This work uncovered a novel function of SALL4/miR-146a-5p axis and its clinical significance in HCC.

Recent studies have highlighted the key involvement of exosomes in remodeling of tumor microenvironment via communication between cancer cells, as well as cancer cells and non-malignant bystander cells such as immune cells, fibroblasts, and endothelial cells. K. Al-Nedawi et al. revealed that epidermal growth factor receptor (EGFR) can be transferred between glioma cells by exosomes, leading to the increased growth ability and the expression of anti-apoptotic genes, indicating an exosomal mediated cancer cell proliferation. In addition, exosomes derived from highly metastatic ovarian cancer strongly induced metastasis in other tumors via exosomal MMP1 mRNA. Furthermore, accumulating evidence supported the oncogenic properties of exosomes in regulating immune cells such as macrophages and neutrophils in several cancer types eg. human head and neck squamous cell carcinoma, mutant p53 cancers, melanoma, and lung cancer. Meanwhile, Wang X, et al. revealed that overexpressing 14–3-3ζ might be transmitted from HCC cells to T cells through exosomes to impair T cell function. It was not surprising that HCC cells are also involved in the process of exosomal transfer. In this study, we confirmed that HCC-derived exosomes can activate macrophages, push macrophages toward M2-like phenotype (Figures 1 and 2), and suppress T cell functions (Figure 3). We also found the effect of macrophages on T cells was mainly influenced by intercellular contacts (data not shown). Majority of the tumor-associated macrophages exhibit M2-like phenotype, but in our study, exosome-educated macrophages shared features of both M1 and M2 types, appearing high levels of IL-1β, TNF-α, CCL17, CCL22, and ARG-1. This was in line with previous studies. Unlike the binary M1/M2 definition, the “educated” macrophages were composed of several distinct populations, but with greater overall similarity to M2-polarized macrophages.

Tumor-derived exosomes could impair T cell functions in different ways. Gautam N et al. considered that tumor-derived exosomes inhibited T cell function directly in head and neck cancer, ovarian tumors, breast cancer, and melanoma, but impaired T cell function by dendritic cells in HCC. However, here we observed HCC-derived exosomes did not influence T cells directly (Figure 3(f)), but they could suppress...
T cell functions by educating macrophages. This inconsistency might be due to the different tumor models or the system and dosage used in our and other studies. In addition, macrophages scavenge cell fragments or protein except membrane fusing with exosomes compared with T cells, and thus can be the superior recipient of exosomes.

Figure 5. Transcription factor SALL4 controlled miR-146a-5p levels in HCC-derived exosomes.

Mice were sacrificed after treatment with DEN/CCL4 for 15 weeks, then hepatocytes and the exosomes from serum were isolated. The mRNA level of sall4 in hepatocytes was analyzed by qPCR (a). CD206+ M2 macrophage number in liver was detected by Flow cytometry (b). MiR-146a-5p in exosomes was analyzed by qPCR (c). RAW264.7 cells were co-cultured with exosomes derived from the serum of DEN/CCL4-treated mice for 24 h, CD206 expression was detected by Flow cytometry (d). CD206+ RAW264.7 cells were co-cultured with exosomes from Hepa1-6 cells transfected with sh-SALL4 or empty vector for 48 h, then CD206+ cells (e), the levels of STAT3 and C/EBPβ (f) were detected by Flow cytometry and western blotting, respectively. The miR-146a-5p level in exosomes derived from Hepa1-6 cells transfected with sh-SALL4 or empty vector was analyzed by qPCR (g). ChIP assay was performed to detect the recruitment of SALL4 in miR-146a-5p promoter (h). 293T cells were co-transfected with pGL-miR146a-Promoter-Luciferase and pcDNA3.1, SALL4 overexpressing plasmid or sh-SALL4 vector, and the Renilla expression vector pRLSV40 was co-transfected to normalize the transfection efficiency. After 24 h (overexpressing SALL4) or 48 h (sh-SALL4), luciferase activity of lysate was detected by dual-GloTM Luciferase assay system. The ratio of firefly and Renilla luciferase activity associated with pGLSV40-Luciferase transfection was set as 1 (i). Exo, exosomes. Data were representative of three independent experiments, statistical significance was determined as ***p < 0.001, **p < 0.01 and *p < 0.05 compared with control.
MiR-146a-5p is a well-known anti-inflammatory miRNA and frequently overexpressed in HCC that played a key role in promoting M2-like phenotype. In the current study, we observed that HCC generated miR-146a-5p-abundant exosomes that transferred miR-146a-5p to macrophages and modulated their polarization and functions (Figure 4). This observation extended the understanding of the oncogenic role of miR-146a-5p except the well-established intracellular signaling of miR-146a-5p. Therefore, oncogenic miR-146a-5p affected not only cancer cells but also tumor microenvironment. Although we focused on miR-146a-5p-abundant exosomes, other microRNAs such as miR-18a, −221, −222, −21 and −224 that are enriched in HCC derived exosomes may also contribute to the recruitment or polarization of immunosuppressive macrophages.

Our previous study suggested that STAT3 could control the expression of miR-146a-5p in human HCC. Here, we found another factor, SALL4, which is reactivated in human HCC and correlates with disease progression of human malignancy and treatment status, regulated miR-146a-5p. Previous study demonstrated that SALL4 to be inseparable from malignant proliferation of tumor cells, and was regulated by the transcription factor STAT3. Here, we found that SALL4 could directly regulate the expression of miR-146a-5p in HCC cells, silencing SALL4 alleviated the generation of miR-146a-5p and M2-polarization (Figure 5), and markedly improved the outcome of HCC in mice (Figure 6). SALL4 is downstream of STAT3, and its binding sequences in the miR-146a-5p promoter overlapped with STAT3 binding sequences. In addition, silencing SALL4 and STAT3 showed similar effects on the transcriptional activity of miR-146a-5p promoter (data not shown). These findings indicated the possibility that SALL4-STAT3 control miR-146a-5p expression cooperatively.

Figure 6. Blocking SALL4 reversed T cell exhaustion and inhibited HCC progression.
Described as Fig. S7A, C57BL/6 mice were treated with DEN/CCL4, meanwhile SALL4 was silenced by hydrodynamic injection of sh-SALL4 vector. The levels of TIGIT, PD-1, and LAG-3 (a), the production of IL-2 and TNF-α, and the expression of NK2GD (b) in T cells from liver of these mice were detected by Flow cytometry. Tumor-related factors afp, ki67 and pcna in hepatocytes were analyzed by qPCR (c). Morphology (d), diameter of the tumor exposed to the liver (e) and H&E staining (f) were analyzed. Data were representative of three independent experiments, statistical significance was determined as ***p < 0.001, **p < 0.01 and *p < 0.05 compared with control.
In conclusion, our findings elucidated that the tumor-macrophage interplay through communication with exosomes during the development of HCC. We uncovered an important role of SALL4-overexpressing hepatoma cell-secreted miR-146a-5p on tumor progression via remolding tumor micro-environment and provided new findings that could potentially contribute to preventing HCC progress.

Materials and methods

Mice

C57BL/6 mice that were purchased from Beijing HFK Bioscience Co., Ltd., were used for all experiments. All animal experiments were carried out in Shandong University (Jinan, China) according to procedures approved by the institutional ethics committee.

Patient samples

Peripheral blood samples of patients were obtained from Qilu Hospital in accordance with the Ethics Committee of Shandong University, informed consent was acquired from all participants.

Cell lines

Mouse HCC cell lines Hepa1-6 cells (Cell Bank of Type Culture Collection of the Chinese Academy of Sciences) and H22 cells (Institute of Basic Medical Sciences, Shandong Academy of Medical Sciences) were cultured with DMEM and RPMI 1640 medium (GIBCO/BRL), respectively. Mouse macrophage cell line RAW264.7 cells and human monocyte cell line THP-1 cells (Cell Bank of Type Culture Collection of the Chinese Academy of Sciences) were cultured in DMEM and RPMI 1640 medium (HyClone), respectively, and 50 mM 2-mercaptoethanol (Sangon) was supplemented for THP-1 cell culture. Human HCC cell line HepG2 cells (Cell Bank of Type Culture Collection of Chinese Academy of Sciences) and H7402 cells (Institute of Basic Medical Sciences, Shandong Academy of Medical Science) were cultured in DMEM and RPMI 1640 medium (GIBCO/BRL), respectively. All cultures were supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin, and maintained in a 5% CO₂ incubator at 37°C.

Peritoneal macrophages (PMs) were extracted from the abdominal cavity of mice with 1× PBS, and cultured with DMEM medium (GIBCO/BRL) supplemented with 10% FBS for 12 h. Then, these cells were washed with 1× PBS and used in follow-up experiments. For the induction of cell differentiation, human THP-1 cells were induced with 100 ng/mL PMA (Sigma, #P1585) for 12 h and rested for another 12 h. For the generation of "exosome-educated" macrophages, PMs, RAW264.7, BMDMs and differentiated THP-1 cells were incubated with 50 μg/mL of HCC-exosomes for 24 h.²⁶

Exosome isolation

Supernatants from HCC cells with over 95% viability and serum of mice were collected, and exosomes were isolated by ultracentrifugation as previously described. Briefly, cell culture medium was sequentially centrifuged at 300 g for 10 min, 2000 g for 30 min and 10,000 g for 70 min at 4°C to remove dead cells and cell debris. Then, the supernatant was filtered through 0.22 mm filter and further ultracentrifuged at 100,000 g for 1.5 h to collect exosomal pellet. The exosome pellets were washed in a large volume of phosphate-buffered saline (PBS) and ultracentrifuged at 100,000 g for another 1.5 h at 4°C. The protein content of the concentrated exosomes was measured using the BCA protein assay kit (Beyotime, #P0010S). Each sample was normalized to a concentration of 2 μg/μl with 1× PBS and stored at −80°C until further use.

Electron microscopy

Exosomes were suspended in 1× PBS and spotted onto formvar-carbon-coated grids (200 mesh), and then the grid was fixed in 2% (vol/vol) paraformaldehyde at room temperature for 5 min. Fixation was followed by washing with deionized water, and then the exosomes were negatively stained using uranyl acetate. Exosomes were visualized under a JEM-1011 transmission electron microscope (JEOL, Japan).

Nanoparticle tracking analysis

Nanoparticles were tracked using a Malvern Zetasizer Nano ZS-90 (Malvern, UK) following the manufacturer’s instructions. Exosomes derived from Hepa1-6 and HepG2 cells were diluted with 1× PBS (1:1000). The mean particle size and size distribution were analyzed by Dynamic Light Scattering (DLS) method through Malvern Zetasizer Nano ZS-90 (Malvern, UK).

Western blot analysis

Cells were lysed with RIPA peptide lysis buffer (Beyotime, #P0013B). Protein concentrations were determined by BCA method and separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, the proteins were incubated with specific antibodies (1:1000). All antibodies are listed in Supplementary Table 2.

Flow cytometry

Harvested cells were incubated with respective antibodies for 40 min at 4°C after washing twice with 1× PBS. For the detection of intracellular markers, cells were fixed, permeabilized, and then stained with respective antibodies for 1 h at 4°C. For intracellular cytokines staining, the cells were pre-treated with 1 μg/mL ionomycin (Beyotime, #S1672) and 50 ng/mL PMA (Sigma, #P1585) for 5 h in the presence of Brefeldin A (BioLegend, #420601) for the last 4 h. The antibodies utilized are listed in Supplementary Table 2. All stained cells were measured using FACS Calibur (BD Biosciences, USA) or
FACS Aria III (BD Biosciences, USA). The data were analyzed with FlowJo software (Treestar Inc., Ashland, OR, USA).

**Detection of reactive oxygen species**

Reactive oxygen species (ROS) were detected by the DCFH-DA assay. Briefly, cells were incubated with 10 μM DCFH-DA (Sigma, #D6883) for 20 min at 37°C. After washing with 1× PBS, ROS production was measured as dichlorofluorescein (DCF) fluorescence intensity by using Olympus IX-71 Inverted microscope (Olympus, Japan).

**Mouse strains and treatments**

Six-week-old C57BL/6 male mice (21–23 g) received HCC-derived exosomes (200 μg/20 g) in 100 μL of 1× PBS via the tail vein every other day. On d 10, liver T cells were harvested for analysis. For macrophage depletion, C57BL/6 mice were injected intravenously with 200 μL of clodronate liposomes (Nicovan RooijenLeb), and depletion efficiency was confirmed by Flow cytometry. BMDMs were transplanted by intravenous administration of 1 × 10⁶ cells, and T cells from liver were harvested 5 d later.

**Isolation of primary T cells**

Lymphocytes were isolated from spleen or liver by Ficoll gradient centrifugation. Primary T cells were purified by CD3 negative-selection isolation kit (Biolegend, #480031) and cultured in RPMI 1640 supplemented with 100 U/mL human rIL-2 (Changsheng).

**T cell proliferation assay**

Hepatic T cells isolated from mice transplanted by intravenous administration with HCC-exosomes-treated BMDMs were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE, Beyotime, #C1031) according to the manufacturer’s instruction, then these CFSE-labeled T cells (5 × 10⁵) were incubated with anti-CD3 (5 μg/mL) and CD28 (2 μg/mL) for 3 d followed by Flow cytometry analysis.

**Cytolysis assay**

CFSE and 7-AAD Viability Staining Solution (Biolegend, #420404) was used to measure the cytolyis activity elicited by T cells against tumor cells. Hepatic T cells from mice transplanted with HCC-exosome-treated BMDMs were pretreated with anti-CD3 (5 μg/mL) and CD28 (2 μg/mL) for 2 d. CFSE-labeled Hepa1-6 cells (2 × 10⁵) were co-incubated with these T cells at different ratios (1:20, 1:10, or 1:5) for overnight. Then, these co-cultured cells were harvested and stained with 7-AAD Viability Staining Solution to confirm by Flow cytometry. The percent lysis was calculated as follows: %lysis = (%CFSE "7AAD")/(%total CFSE ") ×100.

**Chromatin immunoprecipitation**

Chromatin Immunoprecipitation (ChIP) was performed according to the manufacturer’s description (Millipore, #17–10086). The final lysate was used for immunoprecipitation with anti-SALL4 (Abcam, #ab29112) or normal IgG. After overnight incubation with SALL4 antibody at 4°C, protein A/G agarose beads were added to isolate immune complexes. Finally, the DNA was extracted and analyzed by PCR using primers designed around the binding sites on miR-146a-5p promoter (miR146a-pro, 5‘- CCCATGTGTGTTGCTCACACAAAC-3’ and 3‘- ATCCCTAAAACCTACTGCTAA -TCAC-3’). The PCR products were assessed on 2% agarose gels.

**Luciferase reporter gene assay**

For the reporter gene assay, 293T cells were plated at a density of 1 × 10⁴ cells/well in 96-well plates (NEST), and transfected with pGL3-miR-146a-5p-Promoter-Luciferase (200 ng/µl, Addgene, #15091), together with SALL4 overexpressing (over-SALL4) or SALL4 silencing (sh-SALL4) vector in the presence of Lipofectamine™ 2000 (Invitrogen, #11668019). The Renilla expression vector pRL-SV40 was co-transfected to normalize the transfection efficiency. After 24 h or 48 h, cells were washed, lysed and a dual-Gloms Luciferase assay system (Promega, #E1910) was used to measure luciferase activity according to the instruction. The ratio of firefly and renilla luciferase activity with pGL3-SV40-Luciferase was set as 1.

**Statistical analysis**

All data are presented as means ± SD of three or more independent experiments. Statistical comparisons between two groups were performed using a Student’s t-test. One-way ANOVA followed by Tukey’s post-hoc test was used to compare the differences among more than two groups, followed by the Bonferroni post hoc test. GraphPad Software Prism 6.0 was used for statistical analysis. Statistically significant differences were set at *p < 0.05, **p < 0.01.

**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| HCC          | hepatocellular carcinoma |
| SALL4        | Sal-like protein-4 |
| APC          | antigen presenting cells |
| TAMs         | Tumor-associated macrophages |
| MHC          | major histocompatibility complex |
| NO           | nitric oxide |
| NK           | natural killer |
| PMs          | peritoneal macrophages |
| TEM          | transmission electron microscopy |

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**Disclosure of Potential Conflicts of Interest**

The authors declare that they have no competing interests.


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