Aus der Klinik und Poliklinik für Allgemein-, Viszeral-, Tumor- und
Transplantationschirurgie Uniklinik Köln
und
Aus der Chirurgischen Klinik und Poliklinik Großhadern
der Ludwig-Maximilians-Universität München
Direktor: Prof. Dr. med. Karl-Walter Jauch

Tumor-derived exosomes inhibit natural killer cell function in the pre-metastatic niche of pancreatic cancer

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Jiangang Zhao
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Berichterstatter:	Prof. Dr. med. Karl-Walter Jauch
Mitberichterstatter:	PD Dr. med. Susanna Müller PD Dr. med. Hendrik Seeliger
Mitbetreuung durch die promovierten Mitarbeiter:	Prof. Dr. med. Christiane J. Bruns Dr. Yue Zhao
Dekan:	Prof. Dr. dent. med. Reinhard Hickel
Tag der mündlichen Prüfung:	17.10.2019

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Köln

Jiangang Zhao

18.10.2019

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I. ABSTRACT

Introduction: Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal malignancies worldwide. More than 50% of patients are diagnosed with late-stage disease. Exosomes are a group of extracellular vesicles released by different types of cells, containing proteins, nucleic acids and lipids, mediating intercellular communication, and thus affecting physiological and pathological conditions. Tumor-derived exosomes have been shown to induce a pre-metastatic niche in the target organ to promote metastasis.

Methods: We isolated exosomes from cell culture supernatants of a highly metastatic pancreatic cell line L3.6pl and a PDAC patient derived primary cell line TBO368 by ultracentrifugation. Exosomes were characterized by Western blotting, nanoparticle tracking analysis and transmission electron microscopy. The protein content of exosomes was analyzed by mass spectrometry. The potential effects of pancreatic cancer-derived exosomes on NK cells were investigated by immunofluorescence and flow cytometry. The exosomal TGF-β1 levels in serum of patients with PDAC were quantified by ELISA.

Results: We found that adhesion receptors, especially integrins such as integrin αv and integrin β5, which are associated with liver-specific metastases, were enriched in pancreatic cancer-derived exosomes. These exosomes also displayed a variety of immune regulatory factors, such as TGF-β1, Nectin-2 and PVR. Then we co-cultured NK cells with exosomes derived from pancreatic cancer cells. After co-culture, the expression of NKG2D, CD107a, TNF-α and INF-γ in NK cells was significantly downregulated. NK cells also exhibited the decreased level of CD71 and CD98, as well as impaired glucose uptake ability. In addition, NK cell cytotoxicity against pancreatic cancer stem cells was attenuated. Moreover, pancreatic cancer-derived exosomes induced the phosphorylation of Smad2/3 in NK cells. Compared to healthy donors, serum exosomal TGF-β1 was significantly increased in patients with PDAC.

Conclusion: In this study, we show that tumor-derived exosomes are responsible for pre-metastatic niche formation in the liver of PDAC. The inhibitory effects of pancreatic cancer-derived exosomes on NK cells represent a mechanism allowing metastatic tumor cells to escape from NK cell immune surveillance in the pre-metastatic niche. We also demonstrate that serum exosomal TGF-β1 was significantly increased in patients with PDAC. In conclusion, these findings emphasize the immunosuppressive role of pancreatic cancer-derived exosomes and provide new insights into our understanding of NK cell dysfunction in the pre-metastatic niche formation of PDAC.

II. INTRODUCTION

2.1. Pancreatic cancer

2.1.1. Background

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal malignancies worldwide[1]. The majority of patients with pancreatic cancer are diagnosed at an advanced stage and lose the opportunity for curative surgery[2]. Even after R0 resection followed by adjuvant chemotherapy and/or radiotherapy, most patients will eventually have recurrence[3-5]. The Surveillance, Epidemiology and End Results (SEER) Program (https://seer.cancer.gov/statfacts/) reveals that the 5-year overall survival for patients with localized disease is 34.3% between 2008 and 2014 in the United States. For those who present with distant metastases, this drops to merely 2.7%. These daunting statistics indicate that of importance is improving our understanding of the metastatic process to reduce the incidence of metastasis and develop effective therapeutic strategies for PDAC patients.

2.1.2. Metastasis of pancreatic cancer

In 1889, Stephen Paget firstly proposed that the interplay between tumor cells (the ‘seeds’) and the target organ microenvironment (the ‘soil’) promoted organ-specific metastasis formation in breast cancer[6]. From then on, the underlying mechanisms of metastasis have been investigated and discussed for more than one century. Over the past few decades, researchers have shed light on the cellular and molecular events during the process of metastasis.

As an important hallmark of cancer, metastasis is a complex process that propagates tumor cells from the site of origin to distant tissues, also known as the metastatic cascade[7-10]. The metastatic process in PDAC involves the detachment of pancreatic cancer cells from the primary tumor, their intravasation into the bloodstream, their extravasation and entry in the pre-metastatic niche, as well as their survival and colonization to form macroscopic metastases at distant sites[11]. The most common

frequent distant metastatic sites for PDAC include liver, lung and brain[12].

Considered as precursors of metastasis, circulating tumor cells (CTCs) represent tumor cells that are shed from the primary tumor and enter the bloodstream[13]. Rapid technological advances have enabled detection and isolation of CTCs from peripheral blood across a variety of cancer types, including breast, lung, colorectal and pancreatic cancer[14-17]. Birte Kulemann *et al.* reported that CTCs were detected in 67.3% of patients with PDAC (39/58)[18]. Theoretically, CTCs may reach any organ and tissue by circulation. Actually, most of CTCs within the bloodstream will die due to loss of matrix-derived survival signals and hemodynamic shear stress[19, 20]. Therefore, one key step for metastatic cascade is the capability of CTCs to survive and colonize in the new microenvironment of the target organ. It has been well recognized that tumor growth and progression are dependent on the tumor microenvironment. On the contrary, little is known about the dynamic microenvironment of the target organ for metastasis in PDAC.

2.1.3. The hepatic pre-metastatic niche in pancreatic cancer

The term "premetastatic niche" describes the microenvironment in a secondary organ that has been affected by the primary tumor to support tumor growth in advance of tumor cell entry[21]. Many studies have identified the existence of pre-metastatic niches in different organs, such as lymph nodes, lung, liver, bone and brain[22-26]. Liu Y *et al.* summarized six characteristics of the pre-metastatic niche, including immunosuppression, inflammation, lymphangiogenesis, angiogenesis/vascular permeability, organotropism, and reprogramming[27]. The hepatic pre-metastatic niche has been revealed in orthotopic PDAC mouse models[28]. However, the underlying mechanisms of liver pre-metastatic niche formation are still not fully understood in human PDAC.

Metastatic pancreatic cancer cells that enter the liver have to encounter a totally new microenvironment, including different cells and extracellular matrix (ECM). Hepatocytes occupy about 80% of all cells in the liver. Other non-parenchymal cells in

the liver consist of sinusoidal endothelial cells, hepatic stellate cells (HSCs), Kupffer cells (KCs), and lymphocytes[29]. During the process of tumor initiation and progression, phenotype and functions of these cells are strongly influenced by the primary tumor. Among these cells, HSCs are the main driver for liver fibrosis[30]. Upon activation, HSCs are able to transdifferentiate into highly proliferative myofibroblasts. Increased production of growth factors and ECM by myofibroblasts triggers a fibrotic response, enhancing the survival and growth of metastatic tumor cells[31]. It has been revealed that liver metastases in PDAC-bearing mice were infiltrated and surrounded by abundant myofibroblasts[32]. KCs constitute approximately 10% of all liver cells. As resident macrophages in the liver, KCs have the phagocytotic activity and can kill tumor cells through the secretion of cytotoxic molecules, such as tumor necrosis factor (TNF)-α and reactive oxygen species (ROS)[33]. However, under certain conditions, KCs can also produce and secret pro-tumorigenic factors, including hepatocyte growth factors (HGF), vascular endothelial growth factors (VEGF), and matrix metalloproteinases (MMP). These cytokines and growth factors can facilitate tumor cell invasion into the parenchymal space, promote tumor cell proliferation and enhance angiogenesis[34]. Therefore, HSCs and KCs are postulated as two candidates involved in the liver pre-metastatic niche formation[35]. Recently, Jae W. Lee *et al.* reported the activation of signal transducer and activator of transcription 3 (STAT3) signaling and upregulation of serum amyloid A1 and A2 in hepatocytes during early pancreatic tumorigenesis in mouse models. Thereby, hepatocytes were able to induce the accumulation of myeloid cells and fibrosis in the liver, which ultimately favored the survival and colonization of metastatic tumor cells[36]. In addition, intrahepatic lymphocytes and bone marrow-derived cells (BMDCs) also participate in liver pre-metastatic niche formation of PDAC[31].

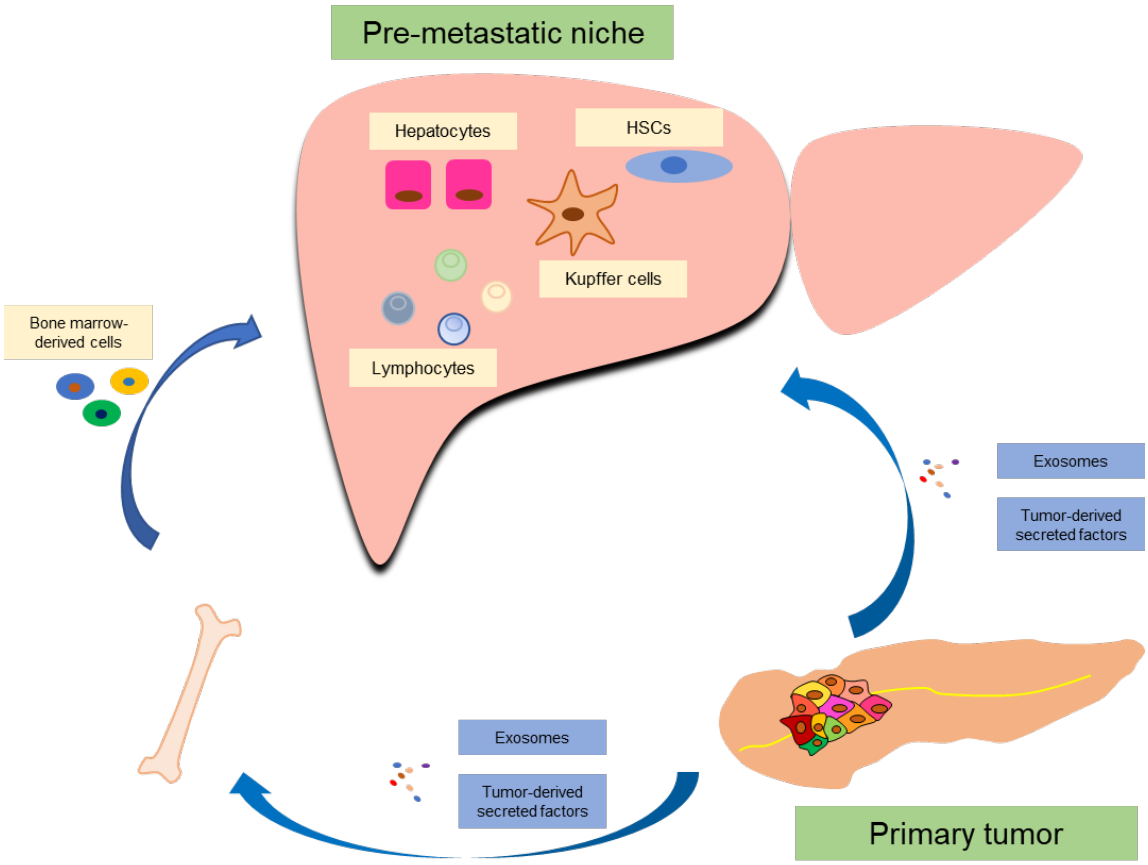

Figure 1. The hepatic pre-metastatic niche in PDAC. Under the influence of the primary tumor, hepatocytes, sinusoidal endothelial cells, hepatic stellate cells (HSCs), Kupffer cells (KCs), lymphocytes and bone marrow-derived cells are involved in liver pre-metastatic niche formation of PDAC.

2.1.4. Cancer immunoediting in the pre-metastatic niche

The human liver is often perceived as a digestive organ, which are responsible for bile production, nutrients storage, synthesis of plasma proteins and detoxification. In fact, the liver also has a large amount of immune cells, including myeloid cells and lymphoid cells[37]. The immune cells in the liver serve as a barrier against metastatic tumor cells. However, the primary tumor can undermine the immune response in the pre-metastatic niche, which ultimately leads to the immune escape of metastatic tumor cells. The concept of cancer immunoediting to explain the dynamics of immune responses in tumor progression is widely accepted in the field of cancer immunology. Cancer immunoediting, both pro-tumorigenic and anti-tumorigenic, is composed of three

processes: elimination, equilibrium and escape[38-40]. Recent advances have begun to uncover the role cancer immunoediting in pre-metastatic niche formation.

In the elimination process, both innate and adaptive immune cells are responsible for cancer immunosurveillance[41]. However, during pancreatic cancer initiation, malignant cells upregulate natural killer cell activating receptor ligands and downregulate inhibitory ligands. For example, major histocompatibility complex class I-related chain A and B (MICA/B) are frequently overexpressed on the surface of pancreatic cancer cells. Such ligands bind to NKG2D receptor to activate natural killer (NK) cells, triggering NK cell cytotoxicity and leading to the secretion of pro-inflammatory cytokines, which regulate other immune cells and facilitate their anti-tumor immune response[42]. In addition, $CD8^+$ T cells can recognize and eliminate pancreatic cancer cells expressing tumor-associated antigens[43]. Therefore, in the elimination process, metastatic pancreatic cancer cells fail to colonize in the pre-metastatic niche.

In the equilibrium process, the immune cell in the liver and pancreatic cancer cells that have survived the elimination process enter into a dynamic equilibrium[44]. Pancreatic cancer stem cells (CSCs) are implicated in metastasis[45]. The quiescent behavior and longevity of pancreatic CSCs makes it easy to accumulate genetic and epigenetic alterations and survive in the equilibrium process[46]. Upon asymmetric division, a metastatic cancer stem cell generates a daughter stem cell for self-renewal and a daughter cell that undergoes further differentiation. The differentiated pancreatic cancer cells are subjected to immunosurveillance and most of them will be detected and destroyed by the immune cells. By contrast, poorly immunogenic tumor cells are more likely to escape from immune surveillance. In addition, the dependence of pancreatic CSCs on their niche also restrain their rapid proliferation[47]. The equilibrium process is functionally similar to the state of tumor dormancy[48]. The tumor cells may stay dormant in the liver for a long time before eventually becoming clinically apparent.

In the escape process, pancreatic cancer cells successfully evade immune destruction. The primary tumor is able to remodel the tumor microenvironment via secretion of immunosuppressive factors and recruitment of immunosuppressive cells, such as regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs), both of which can attenuate the cytotoxicity of $CD8^+$ T cells and NK cells[49-51]. Besides, during tumor progression, HSCs and KCs also promote immunosuppression in the liver. The immunosuppressive microenvironment in the pre-metastatic niche allows pancreatic cancer cells to rapidly proliferate. Finally, metastatic pancreatic cells and their differentiated progeny progressively grow into a visible metastasis in the liver.

Taken together, the mechanisms by which the primary tumor affects the process of cancer immunoediting may explain the immune escape of tumor cells in the hepatic pre-metastatic niche of PDAC.

2.2. Natural killer cells

2.2.1. Background

Natural killer (NK) cells are a group of innate lymphocytes, which are able to recognize and eliminate virus-infected cells and malignant cells. In addition to cytotoxicity, NK cells also secrete numerous cytokines and chemokines to influence the immune system[52]. Several activating and inhibitory receptors regulating NK cell function have been identified (Table 1)[53]. Among these receptors, NKG2D is one of the best characterized activating receptors. It is a type II transmembrane protein with a C-type lectin-like extracellular domain expressed on the surface of NK cells[54]. NKG2D can recognize and bind a diverse array of ligands. Among them, MICA, MICB, ULBP-4 and ULBP-5 are transmembrane-anchored proteins, whereas ULBP-1, ULBP-2, ULBP-3 and ULBP-6 are glycophosphatidylinositol–anchored proteins[55]. Upon receptor-ligand interaction, NKG2D phosphorylates DAP10 or DAP12, recruits and activates phosphatidylinositol 3 kinase, thus triggering NK cell cytotoxicity[52].

Table 1. Activating/inhibitory receptors of NK cells and their ligands

Activating receptors	Ligands	Inhibitory receptors	Ligands
NKG2C	HLA-E	CD96	NECTIN2
NKG2D	MICA		PVR
	MICB	TIGIT	NECTIN2
	ULBP1-6		PVR
NKp46	Heparan sulfates	PVRIG	NECTIN2
	VIM		PVR
	Viral HA	PD-1	PD-L1
NKp44	Heparan sulfates		PD-L2
	KMT2E	LAG3	MHC Class II
	PCNA	NKG2A	HLA-E
	Viral HA		
NKp30	BAG6		
	Heparan sulfates		
	NCR3LG1		

2.2.2. NK cells and tumor

NK cells have the capability to detect and kill tumor cells independent of antigen recognition, which is different from $CD8^+$ T cells[56]. There are two manners for NK cells to eliminate cells. On the one hand, special ligands on the surface of tumor cells can bind to activating receptors on NK cells, which ultimately activates NK cell cytotoxicity[57]. On the other hand, the Fc portion of immunoglobulins on antibody-coated tumor cells can bind to the FcγRIII on NK cells, leading to antibody-dependent cellular cytotoxicity (ADCC)[58]. In addition, NK cells can connect innate and adaptive immune system through secretion of immunomodulatory cytokines. Recently, NK cells are reported to harbor properties of adaptive immunity and abilities to maintain immunological memory[59].

The role of NK cells in PDAC has received less attention but is increasingly being

recognized. Gürlevik E *et al.* reported that after primary tumor resection, gemcitabine treatment triggered NK cell cytotoxicity against tumor cells and decreased local recurrence incidence in orthotopic PDAC mouse models[60]. Ames E *et al.* found that NK cells preferentially killed pancreatic CSCs *in vitro* and intratumoral injection of NK cells in the human pancreatic cancer-bearing NSG mice reduced the percentage of pancreatic CSCs and tumor burden [61]. Therefore, immunotherapies based on NK cells are attracting more attention in PDAC.

However, the ability to escape from immune surveillance has been established as a hallmark of tumor cells[10]. Increasing studies have revealed NK cells exhaustion in tumor[62]. NK cells in cancer patients produced decreased cytokines, downregulated activating receptors, as well as exhibited impaired cytolytic activity[63-65]. In addition, dysregulated cellular metabolism has been observed in dysfunctional NK cells[66]. Cong J *et al.* found that in lung cancer mouse models, the expression of gluconeogenesis enzyme fructose bisphosphatase 1 (FBP1) was upregulated in tumor-infiltrating NK cells, which mediates dysfunction of NK cells by impairing glycolysis[67]. The mechanisms for NK cell dysfunction include direct inhibition via cell–cell contact, and indirect inhibition, via the production of inhibitory factors, such as TGF-β1, IL-10, PGE2 and IDO[68, 69].

2.2.3. NK cells in the pre-metastatic niche

NK cells also play a non-negligible role in the control of metastasis[70]. In solid tumors, impaired NK cell function was associated with the incidence to develop metastases[71]. NK cells occupy about 5%-15% of lymphocytes in the peripheral blood. By contrast, in the liver, NK cells constitute around 30%-40% of intrahepatic lymphocytes[72]. During the metastatic process, when the metastatic pancreatic cancer cells enter the liver, their survival is largely dependent on their successful escape from NK cell immunosurveillance. Recent studies have identified the existence of an immunosuppressive microenvironment in the pre-metastatic niche[27]. However, the dynamics of NK cell in the hepatic pre-metastatic niche of PDAC still remains unknown.

2.3. Exosomes

2.3.1. Background

Cells can secrete different types of extracellular vesicles (EVs) to communicate with neighboring and distant cells[73]. In general, EVs can be classified as microvesicles and exosomes based on their size, as well as their mechanisms of biogenesis and release. Microvesicles, 100 to 1,000 nm in diameter, are generated by budding at the plasma membrane. By contrast, ranging from 30 to 100 nm in diameter, exosomes are formed after fusion of multivesicular endosomes/multivesicular bodies with the cell membrane[73, 74]. Through the transfer of various cargos, including proteins, nucleic acids (DNA, mRNA, microRNA, etc.) and lipids, exosomes can mediate intercellular communication, and thus affect physiological conditions[75, 76]. Moreover, exosomes are linked to a variety of diseases, including neurodegenerative diseases, disorders of the immune system, cardiovascular diseases and cancer[77-80].

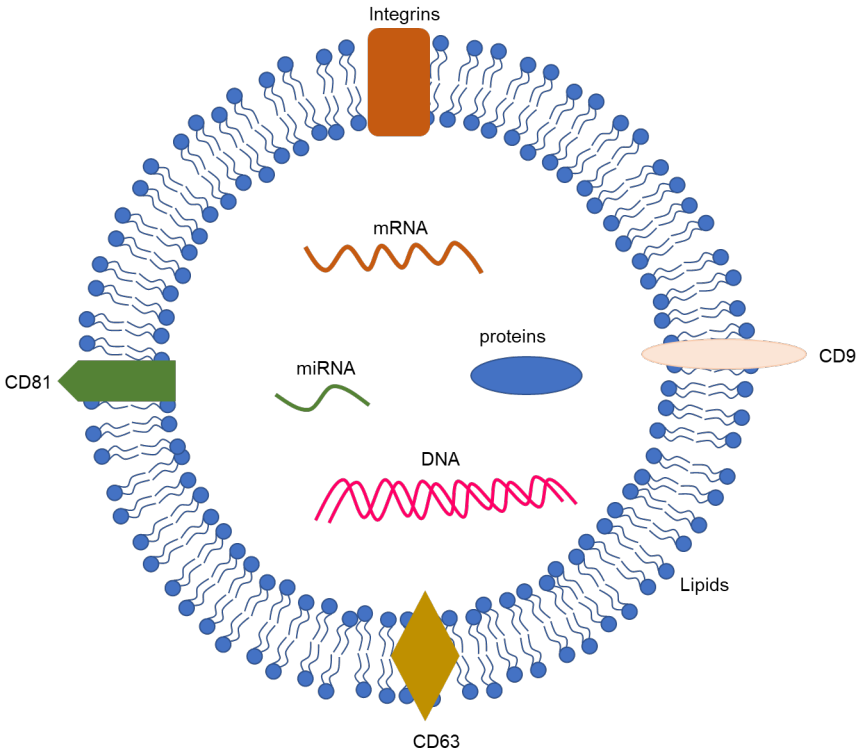

Figure 2. Molecular composition of exosomes. Exosomes are composed of a lipid bilayer loaded with proteins, nucleic acids (DNA, microRNAs, mRNA) and lipids. As a mediator, exosomes can transfer signals and information to neighboring and distant

sites. CD9, CD63, CD81 are tetraspanin proteins in exosomes and often regarded as exosomal markers.

2.3.2. Exosomes in pancreatic cancer

Exosomes exist in various body fluids, such as serum, breast milk and saliva[81-83]. Recently, they have become potential non-invasive biomarkers in early diagnosis as well as prediction of treatment effect and prognosis in cancer patients. For example, Melo SA *et al.* reported that serum glypican-1 positive exosomes could be developed to distinguish patients with PDAC from healthy individuals and patients with benign pancreatic diseases. Levels of glypican-1 positive exosomes in serum correlated with tumor burden and the survival of PDAC patients[84]. Allenson K *et al.* found that compared to circulating cell-free DNA, patients with localized PDAC exhibit a higher percentage of detectable KRAS mutations in serum exosomes. This finding indicated that circulating exosomal KRAS mutation might be developed as a screen tool for early detection of PDAC.[85].

In addition, as lipid bilayer membrane vesicles, exosomes are one ideal carrier for drug delivery in cancer treatment[86]. Kamerkar S *et al*. modified exosomes released by fibroblast-like mesenchymal cells to deliver short interfering RNA specific to KRAS mutation. Treatment of these engineered exosomes suppressed tumor growth in PDAC-bearing mice and significantly increased their overall survival[87].

In general, tumor cells are thought to synthesize more proteins, nucleic acids and lipids[88]. Exosomes secretion has been proposed as a mechanism to maintain cellular homeostasis by removing excess molecules from tumor cells[89]. Consequently, tumor-derived exosomes containing these cargos can be taken up by neighboring cells, as well as enter the bloodstream and travel to distant sites. Desmoplasia is a common feature for the tumor microenvironment of PDAC, which consists of pancreatic cancer cells, cancer associated fibroblasts (CAFs), tumor-associated macrophages (TAMs), pancreatic stellate cells (PSCs), endothelial cells and various types of immune cells, as well as the extracellular matrix (ECM) that are produced by these cells[90]. The

interactions between the tumor cells and the tumor microenvironment are crucial for tumor initiation and progression. Tumor-derived exosomes have the capability to modulate the stromal cells to establish a favorable microenvironment that supports tumor growth. Masamune A *et al.* found that exosomes derived from pancreatic cancer cells induced the activation of PSCs[91]. Activated PSCs secreted a large amount of growth factors, chemokines, and ECM constituents to facilitate tumor growth and invasion[92]. Tumor growth is also dependent greatly on angiogenesis to supply nutrients and oxygen[93]. Zeng Z *et al.* reported that exosomes released from pancreatic cancer cells regulated gene expression in human umbilical vein endothelial cells (HUVECs) and induced angiogenesis *in vitro*[94]. Taken together, tumor-derived exosomes mediate intercellular communication within the tumor microenvironment of PDAC.

2.3.3. The role of exosomes in pre-metastatic niche formation

Tumor-derived exosomes can be released into the circulation and transferred to distant sites. Recent research has shed light on the role of tumor-derived exosomes in pre-metastatic niche formation. For instance, exosomes released by colorectal cancer (CRC) cells could increase vascular permeability and promote liver and lung metastasis formation in mouse models[95]. In murine models of lung cancer and melanoma, tumor-derived exosomes delivered signals to lung epithelial cells and activated Toll-like receptor 3 (TLR3), which elicited chemokine production and promoted neutrophil infiltration[96]. PDAC-derived exosomes recruited macrophages and neutrophils to the liver, and stimulated HSCs to synthesize fibronectin to promote liver metastasis[97]. In addition, exosomal integrins could determine organotropic metastasis[98]. In conclusion, tumor-derived exosomes are involved in pre-metastatic niche formation, including angiogenesis, immunosuppression and organotropism.

2.4. Summary for this part

In this study, we have investigated the effect of tumor-derived exosomes on NK cells

in pancreatic cancer. Here, we provide evidence that pancreatic cancer-derived exosomes carry abundant immunosuppressive factors and inhibit NK cell function, which contributes to pre-metastatic niche formation.

III. MATERIALS AND METHODS

3.1.Materials

3.1.1. Cell lines

3.1.1.1.Human pancreatic cancer cell line L3.6pl

L3.6pl is a highly metastatic human pancreatic cancer cell line variant originally derived from fast-growing variant (FG) of COLO375 after several cycles of *in vivo* selection. In orthotopic xenograft mouse models, L3.6pl cells exhibit an aggressive tumor growth with multiple spontaneous lymph node metastases and liver metastases[99].

3.1.1.2.Human pancreatic cancer cell line TBO368

TBO368 was isolated from the primary tumor of a patient with PDAC from the Department of General, Visceral and Cancer Surgery, University Hospital of Cologne. The study has been approved by the Ethics Committee of the University of Cologne (BIOMASOTA (Biologische Material Sammlung zur Optimierung Therapeutischer Ansätze), ID: 13-091, approval in May 2016). To avoid the contamination of fibroblasts and immune cells, differential trypsinization was conducted until a homogenous population of EpCAM-expressing human pancreatic cancer cells was achieved by flow cytometry. In addition, we injected TBO368 cells subcutaneously into 6 to 8 week-old female NSG mice to verify their *in vivo* tumorigenicity. TBO368 cells were expanded and stored within 20 passages for future experiments.

3.1.1.3.Mouse pancreatic cancer cell line Panc02

Pan02 cells were developed from C57BL/6 mice treated with 3-methyl-cholanthrene. Pan02 cells are resistant to many standard chemotherapy applied in patients with PDAC[100]. Both orthotopic and intrasplenic injection of Panc02 cells resulted in multiple liver metastases in C57BL/6 mice[101].

3.1.1.4.Human natural killer cell line NK-92

NK-92 is a natural killer cell line isolated from the peripheral blood of a 50-year old patient with rapidly progressive non-Hodgkin's lymphoma[102]. The growth of NK-92 cells is dependent on the presence of interleukin-2 (IL-2). NK-92 cells are cytotoxic to a wide range of human tumor cells, including pancreatic cancer cells[103].

3.1.2 Materials for cell culture

DMEM low Glucose	Cat# 12320032, Gibco Invitrogen, Germany
DMEM high Glucose	Cat# 41965062, Gibco Invitrogen, Germany
DMEM/F12	Cat# 31331093, Gibco Invitrogen, Germany
Advanced DMEM/F-12	Cat# 12634028, Gibco Invitrogen, Germany
RPMI 1640 + Glutamax-1	Cat# 61870044, Gibco Invitrogen, Germany
MEM α, no nucleosides	Cat# 22561021, Gibco Invitrogen, Germany
Fetal bovine serum (FBS)	Cat#FBS12-A, Capricorn Scientific GmbH, Germany
Horse serum	Cat# 16050122, Gibco Invitrogen, Germany
Fetal Bovine Serum, exosome-depleted	Cat# A2720803, Gibco Invitrogen, Germany
DPBS	Cat# P04-36500, PAN Biotech, Germany
Penicillin/Streptomycin (10,000 Units Penicillin/mL, 10 mg Streptomycin/mL)	Cat# 15140122, Gibco Invitrogen, Germany
Trypsin 0.05%/EDTA 0.02 % in PBS without Ca^{2+} and Mg^{2+}	Cat# 25300054, Gibco Invitrogen, Germany
EGF (Recombinant human EGF)	Cat# AF-100-15, PeproTech, UK
bFGF (Recombinant human basic FGF)	Cat# 100-18B-250, PeproTech, UK
Insulin solution (human)	Cat# I9278, Merck, Germany
B27 (B-27™ Plus Supplement (50X))	Cat# A3582801, Gibco Invitrogen, Germany
rhIL-2 (Recombinant Human IL-2)	Cat# 200-02, Peprotech, USA
MEM vitamin mixture	Cat# 11120052, Gibco Invitrogen, Germany
MEM Non-Essential Amino Acids (NEAA)	Cat# 11140035, Gibco Invitrogen, Germany

L-Glutamine 200 mM (100X)	Cat# 25030123, Gibco Invitrogen, Germany
Normocin	Cat# ant-nr-2, InvivoGen, San Diego, USA
Trypan blue stain (0.4%)	Cat# T10282, Invitrogen, Germany
DMSO	Cat#, A36720100, AppliChem, Germany
PKH67	Cat# MINI67-1KT, Merck, Germany

3.1.3 Medium for cell culture and cryopreservation

3.1.3.1 Cell culture medium for pancreatic cancer cell lines

Cell line	Medium	Supplements
L3.6pl	DMEM low	10% FBS
		1% MEM vitamin mixture
		1% MEM NEAA
		2 mM L-Glutamine
		100 IU/mL Penicillin
		100 µg/mL Streptomycin
TBO368	Advanced DMEM/F-12	10% FBS
		2 mM L-Glutamine
		100 IU/mL Penicillin
		100 µg/mL Streptomycin
Panc02	RPMI 1640 + Glutamax-1	10% FBS
		100 IU/mL Penicillin
		100 µg/mL Streptomycin

3.1.3.2 Cell culture medium for natural killer cell line

Cell line	Medium	Supplements
NK-92	MEM α, no nucleosides	12.5% FBS
		12.5% Horse serum
		100 IU/mL Penicillin
		100 µg/mL Streptomycin
		0.02 mM Folic acid
		0.1 mM 2-mercaptoethanol
		0.2 mM Myo-inositol

		2 mM L-Glutamine
		100 IU/mL rhIL-2

3.1.3.3 Medium for tumor sphere formation assay

Tumor spheres	DMEM/F12	20 ng/mL EGF
		20 ng/mL bFGF
		5 µg/mL insulin
		1X B27

3.1.3.4 Cryopreservation medium

90% FBS+10% DMSO

3.1.4 Materials for exosomes isolation

3.1.4.1 Exosomes isolation from cell culture supernatants

Polycarbonate Bottle with Cap Assembly	Cat# 355603, Beckman Coulter, USA

3.1.4.2 Exosomes isolation from serum

ExoQuick Exosome Precipitation Solution for Serum	Cat# EXOQ5A-1-SBI, System Biosciences, Germany

3.1.5 Real-Time Quantitative PCR

3.1.5.1 Materials for Real-Time Quantitative PCR

RNeasy Mini Kit	Cat# 74106, QIAGEN, Germany
High-Capacity cDNA Reverse Transcription Kit	Cat# 4368814, Applied Biosystems, USA
Fast SYBR™ Green Master Mix	Cat# 4385612, Applied Biosystems, USA
MicroAmp™ Optical 96-Well Reaction Plate	Cat# N8010560, Applied Biosystems, USA
MicroAmp™ Clear Adhesive Film	Cat# 4306311, Applied Biosystems, USA

3.1.5.2 Primer sequences used for PCR

5’-3’

hKras exon2 seq-for	TGAAGTACAGTTCATTACGATACACG
hKras exon2 seq-rev	GGAAAGTAAAGTTCCCATATTAATGGT
MICA-for	CTGTGCCCTCTGGGAAAGTG
MICA-rev	CGTCCCAACTGGGTGTTGAT
MICB-for	CCTGTGCCCTCTGGGAAG
MICB-rev	GTGGTCTCCTGTCCCAACTG
ULBP1-for	TGGCAGATGAGGAGAGTTGTTTA
ULBP1-rev	TGTTGAGCCGACAATGTCCT
ULBP2-for	AAGTGCAGGAGCACCACTC
ULBP2-rev	TGCTCACAGGAGCCTTTTGG
ULBP3-for	AAGAGCTGGCTTAGGGACTTC
ULBP3-rev	TATCACCTTCCACCTGTCACTC

3.1.6 Materials for Western blot (WB)

3.1.6.1 Reagents and Consumables for Western blot

cOmplete™ Lysis-M	Cat# 4719956001, Merck, Germany
cOmplete™ ULTRA Tablets, Mini, EASYpack Protease Inhibitor Cocktail	Cat# 5892970001, Merck, Germany
PhosSTOP™	Cat# 4906845001, Merck, Germany
Pierce™ BCA Protein Assay Kit	Cat# 23225, Thermo Scientific™, Germany
Pierce™ LDS Sample Buffer, Non-Reducing (4X)	Cat# 84788, Thermo Scientific™, Germany
Roti®-Block, 10X	Cat# A151,2, Carl Roth, Germany
SuperSignal™ West Pico PLUS Chemiluminescent Substrate	Cat# 34577, Thermo Scientific, Germany
Western Blotting Filter Paper, Extra Thick, 8.5 cm x 9 cm	Cat# 88610, Thermo Scientific, Germany
PVDF membrane 0.2µM	Cat# 741260, MACHEREY-NAGEL, Germany
PageRuler™ Prestained Protein Ladder 10 to 180 kDa	Cat# 26617, Thermo Scientific, Germany

3.1.6.2 Buffer systems for Western blot

Running buffer (Tris-Glycine/SDS), pH 8.3

25 mM Tris

190 mM Glycine

0.1% SDS

ddH_2O

Transfer buffer, pH 8.3

25 mM Tris

190 mM Glycine

20% Methanol

ddH_2O

Washing buffer (TBST)

20 mM Tris

150 mM Sodium Chloride

0.1% Tween-20

ddH_2O

Adjust pH to 7.4–7.6 with HCl

Antibody dilution solution

1X Roti-Block in H_2O

3.1.6.3 Antibodies for Western blot

Antibody	Company	Catalog	Specificity	Host
CD9	System Biosciences	EXOAB-KIT-1-SBI	human	rabbit
CD63	System Biosciences	EXOAB-KIT-1-SBI	human	rabbit
CD81	System Biosciences	EXOAB-KIT-1-SBI	human	rabbit
Alix	Santa Cruz	sc-53540	human	mouse
TSG101	Santa Cruz	sc-136111	human	mouse

Flotillin-1	Santa Cruz	sc-74566	human	mouse
Rab5	Santa Cruz	sc-46692	human	mouse
TGF-β1	Abcam	ab64715	human	mouse
Nectin-2	Proteintech	27171-1-AP	human	rabbit
PVR	Proteintech	27486-1-AP	human	rabbit
ITGAV	abcam	Ab179475	human	rabbit

3.1.7 Materials for mass spectrometry (MS)

50x Protease Inhibitor cocktail	Ref# 11873580001, Merck, Germany
Triethylammoniumbicarbonate (TEAB)	Ref# T7408, Sigma, Germany
Urea	Ref# U1250, Sigma, Germany
Benzonase HC nuclease	Ref# 71206-3, Merck, Germany
Dithiothreitol (DTT)	Ref# A1101, AppliChem, Germany
2-Chloroacetamide	Ref# 79-07-2, Merck, Germany
Trypsin	Ref# 9002-07-2, Serva, Germany
Lysyl Endopeptidase (Lys-C)	Ref# 129-02541, WAKO Chemicals GmbH, Germany
Formic acid	Ref# 94318, Honeywell/ Fluka, Romania
Acetonitrile	Ref# 1000291000, Merck, Germany
Methanol	Ref# 83638.32, VWR, Germany
MS grade water	Ref# 270733, Merck, Germany

3.1.8 Materials for flow cytometry

3.1.8.1 Reagents for flow cytometry

TruStain FcX™ (anti-mouse CD16/32)	Cat# 101319, Biolegend, USA
Cell Staining Buffer	Cat# 420201, Biolegend, USA
Human TruStain FcX™ (Fc Receptor Blocking Solution)	Cat# 422301, Biolegend, USA
Red Cell Lysis Buffer	Cat# 158904, Qiagen, Germany
UltraComp eBeads™ Compensation	Cat# 01-2222-42, Invitrogen™, Germany

Beads	
Zombie Aqua# Fixable Viability Kit	Cat# 423102, Biolegend, USA
Intracellular Staining Permeabilization Wash Buffer (10X)	Cat# 421001, Biolegend, USA
Polybead® Carboxylate Microspheres 4.50μm	Cat# 17140-5, Polysciences, USA
Brefeldin A Solution (1,000X)	Cat# 420601, Biolegend, USA
Monensin Solution (1,000X)	Cat# 420701, Biolegend, USA
2-NBDG	Cat# 11046-1, Cayman

3.1.8.2 Antibodies for flow cytometry

Antibody	Company	Cat#
FITC anti-mouse CD45	Biolegend	103107
Alexa Fluor® 700 anti-mouse CD3	Biolegend	100215
PerCP/Cy5.5 anti-mouse CD19	Biolegend	152405
PE/Dazzle# 594 anti-mouse NK-1.1	Biolegend	108747
Brilliant Violet 421# anti-mouse CD335	Biolegend	137611
APC anti-mouse CD49a	Biolegend	142605
PE/Cy7 anti-mouse CD49b	Biolegend	103517
Brilliant Violet 421™ anti-human CD314 (NKG2D)	Biolegend	320821
FITC anti-human CD45	Biolegend	304005
APC anti-human CD107a (LAMP-1)	Biolegend	328619
PE/Dazzle™ 594 anti-human TNF-α	Biolegend	502945
PE/Cy7 anti-human IFN-γ	Biolegend	506517
FITC anti-human CD98	Biolegend	315603
PE anti-human CD71	Biolegend	334105
APC anti-human MICA/MICB	Biolegend	320907
PE Mouse anti-Smad2 (pS465/pS467)/Smad3 (pS423/pS425)	BD Bioscience	562586

3.1.9 Materials for immunofluorescence (IF) and immunohistochemistry (IHC)

3.1.9.1 Reagents for IF and IHC

Normal Serum Block	Cat# S3023, Dako, USA
Fluorescence mounting medium	Cat# S3023, Dako, USA
DAPI	Cat# D1306, Invitrogen™, Germany
Dako EnVision+ System, HRP (AEC), For use with mouse primary antibodies	Cat# K4005, Dako, USA
Dako EnVision+ System, HRP (AEC), For use with rabbit primary antibodies	Cat# K4009, Dako, USA
Antibody diluent reagent solution	Cat# 005218, Life technologies, USA
Hydrogen Peroxide 30%	Cat# CP26.5, Carl Roth, Germany
Tissue-Tek O.C.T.TM.	Cat# 25608-930, VWR, Germany
Polysine Adhesion Slides	Cat# J2800AMNT, Thermo Scientific™, Germany

3.1.9.2 Buffer systems for antigen retrieval

Tris/EDTA pH 9.0	10 mM Tris 144 mM Sodium Chloride Adjust pH to 9.0, with HCl
Citric acid pH 6.0	10 mM Citric acid Adjust pH to 6.0, with Sodium Hydroxide

3.1.10 Materials for ELISA

TGF beta-1 Human/Mouse Uncoated ELISA Kit	Cat# 88-8350-88, Invitrogen, Germany
Wash buffer	PBS wash buffer with 0.05% Tween-20
Reagent diluent	1% BSA in PBS
Substrate solution	TMB Substrate
Stop solution	H_2SO_4
Streptavidin-HRP	

3.1.11 Animal experiments

3.1.11.1 Animals

C57BL/6 mice, 6-8 weeks, female	*In vivo* Research Facility, CECAD Cologne, Germany
NSG mice, 6-8 weeks, female	*In vivo* Research Facility, CECAD Cologne, Germany

3.1.11.2 Surgical instruments

Forceps	Dosch GmbH, Heidelberg, Germany
Disposable scalpels	Feather Safety Razor Co., Japan
Scissors, sharp / blunt	Dosch GmbH, Heidelberg, Germany

3.1.11.3 Medicine

Ketaminhydrochlorid (Ketavet), 100 mg/mL	Pfizer Pharmacia GmbH, Germany
Xylazinhydrochlorid, Xylazin (Rompun) 2%, 25 mL	Bayer Healthcare, Germany

3.1.11.4 Other materials

Syringe (1mL, 5 mL, 10 ml)	B Braun, Germany
Injection needle (26G, 28 G)	B Braun, Germany
Thread with needle USP 4/0 Seralon®	Serag-Wiessner AG, Naila, Germany
Rotilabo®-embedding cassettes	Cat# K116.1, Carl Roth, Germany
Roti®-Histofix 4 %	Cat# P087.3, Carl Roth, Germany
Percoll™	Cat# 17-0891-02, VWR, Germany
PBS (10X), pH 7.4	Cat# 70011044, Gibco Invitrogen, Germany

3.1.11.5 Percoll solution for intrahepatic lymphocytes isolation

100% Percoll	10X PBS	Percoll
1 mouse	2.25 mL	20.25 mL
70% Percoll	100% Percoll	RPMI
1 mouse	10.95 mL	4.7 mL
45% Percoll	100% Percoll	RPMI

1 mouse	2.8 mL	3.45 mL

3.1.12 Chemicals

Albumin Fraction V	Cat# 8076.2, Carl Roth, Germany
Tris	Cat# 9127.2, Carl Roth, Germany
Glycine	Cat# 1313400914, AppliChem, Germany
SDS	Cat# A72495000, AppliChem, Germany
Methanol	Cat# 4627.5, Carl Roth, Germany
Sodium Chloride	Cat# 3957.2, Carl Roth, Germany
Tween 20	Cat# 9127.2, Carl Roth, Germany
Xylene	Cat# 371.5000, Th. Geyer, Germany
99% 2-Propanol	Cat# 9866.5, Carl Roth, Germany
96% ethanol	Cat# 22065000, Th. Geyer, Germany
EDTA	Cat# E-5134, Merck, Germany
Citric acid monohydrate	Cat# 100244.0500, Merck, Germany

3.1.13 Technical equipment

Biological Safety Cabinets Class II	Herasafe KS, Thermo Scientific™, Germany
	NU-440-400E, NUAIRE, USA
Air-displacement pipettes	Eppendorf, Germany
CO_2- incubators	Heracell 150i, Thermo Scientific™, Germany
	MCO-230AICUV-PE, Panasonic, Japan
Centrifuges	Megafuge 1.0R, Heraeus, Germany
	Megafuge 40R, Heraeus, Germany
	Heraeus™ Fresco™ 17 Microcentrifuge, Thermo Scientific™, Germany
Phase Contrast Microscope	DFC450C, Leica, Germany
Automated Cell Counter	Countess II, Invitrogen, USA
Vortex	Lab dancer, VWR, Germany
Refrigerator 4°C	Liebherr, Germany
Freezer -20°C	Bosch, Germany
Freezer -80°C	Sanyo, Japan

Freezer -150°C	Sanyo, Japan
Cell freezing container	Cell Camper Mini-12, neoLab, Germany
Water bath	Störk-Tronic, Germany
Ultracentrifuge	Optima™ L-90K, Beckman Coulter, USA
Rotor for Heraeus Megafuge 40R	HIGHConic SN999, Thermo Scientific™, Germany
Plate Reader	FLUOstar Omega, BMG Labtech, Germany
Mini-PROTEAN® System	Bio-Rad, USA
Trans-Blot® Turbo™ Transfer System	Bio-Rad, USA
INTAS ECL CHEMOSTAR	Intas Science Imaging, Germany
Microtome	Techno-Med GmbH, Germany
Cryostat	Thermo Scientific™, Germany
PT-Module	Cat# A80400011, Thermo Scientific™, Germany
Fluorescent Cell Imager	InCellis, Bertin, France
Thermocycler	Tpersonal, Biometra, Germany
Thermomixer	ThermoMixer C, Eppendorf, Germany
Spectral Analyzer	NanoDrop One, Thermo Scientific™, Germany
Real time PCR	QuantStudio 7 flex, Applied Biosystems, USA
Spectrometer	Direct Detect®, Merck, Germany
Mass Spectrometer	QExactive Plus/ Easy nLC 1200, Thermo Scientific™, Germany

3.1.14 Consumables

15 mL centrifuge tubes	Sarstedt, Germany
50 mL centrifuge tubes	Sarstedt, Germany
6, 12, 24 and 96-well cell culture plates	Sarstedt, Germany
Serological pipettes 5mL	Sarstedt, Germany
Serological pipettes 10mL	Sarstedt, Germany
Serological pipettes 25mL	Sarstedt, Germany
Pipette tips 10µL	Sarstedt, Germany

Pipette tips 200µL	Sarstedt, Germany
Pipette tips 1000µL	Sarstedt, Germany
Tissue culture flask T-25	Sarstedt, Germany
Tissue culture flask T-75	Sarstedt, Germany
Tissue culture flask T-150	Sarstedt, Germany
Cryotubes 1.8 mL	Sarstedt, Germany
Sterile reaction tube	Sarstedt, Germany
Cell counting slide	Cat# 734-2676, VWR, Germany
Ultra-Low Attachment 6-Well Plate	Cat# CLS3471, Corning, USA,
Cell strainer, 40 µm pore size, blue	Cat# 734-2760, VWR, Germany
Cell strainer, 70 µm pore size, blue	Cat# 734-2761, VWR, Germany

3.1.15 Software

Acrobat Reader DC	Professional Adobe Systems Inc., USA
Microsoft Office 365 (Word, Excel, PowerPoint)	Microsoft Corporation, USA
ImageJ	National Institutes of Health
Graphpad Prism 8	GraphPad Software, Inc., USA
EndNote X7	Thomson Reuter, CA, USA
FlowJo software	Treestar Inc., Ashland, USA
Windows 10	Microsoft Corporation, USA

3.2 Methods

3.2.1 Cell culture conditions

All cell lines were cultured in 25, 75 or 175 cm^2 flasks and incubated at 37 °C in a humidified incubator with 5% CO_2. The culture medium was replaced every 2-3 days. Pancreatic cancer cells were passaged with 0.05% Trypsin-EDTA when 80-90% confluency was reached. NK-92 cells were cultured in suspension at 0.2-0.6 × 10^6 cells/mL. Saturated NK cell culture was passaged 1:2 every 2-3 days.

3.2.2 Determination of cell number and storage of cells

The cell number was determined using Countess II Automated Cell Counter (Invitrogen). Briefly, 10 µL of cells and 10 µL of trypan blue solution were mixed

thoroughly. Then 10 μL of mixture were pipetted into a dispensable countess chamber slide. The slide was inserted into Countess II Automated Cell Counter. The concentration of cells was obtained directly from the display. Usually, 1 to 4 million cells were centrifuged at 350 g for 5 min. The supernatant was discarded and the pellet was resuspended in 1 mL of cryopreservation medium. The tubes were put into a cell freezing container (neoLab) and then kept in the -80°C freezer overnight. Next day, the tubes were transferred into the -150°C freezer for storage.

3.2.3 Recultivation of cells

The appropriate medium was put into a new cell culture flask. The cryopreservation tube was taken out from the -150°C freezer. Then the tube was immediately transferred into the 37°C water bath and thawed for 1-2 min. After complete thawing, the tube was taken out and centrifuged at 350 g for 5 min. After centrifugation, the supernatant was discarded. The pellet was resuspended in 1 mL of culture medium and transferred into the cell culture flask.

3.2.4 Establishment of orthotopic PDAC mouse models

Animal experiments were conducted according to protocols approved by the responsible national and local authority (81-02.04.2018.A139, LANUV NRW, approved on 20th September 2018). All mice were housed under pathogen-free conditions with unrestricted diet and water under a 12:12 h light/dark cycle. Female C57BL/6 mice, 6 to 8 weeks of age, were used to establish orthotopic pancreatic cancer mouse models. After general anesthesia, shaving, disinfection and abdominal cavity opening, the spleen was taken out with the pancreas tail. Generally, 1×10^6 Panc02 cells were injected into the pancreas tail. After injection, the spleen and pancreas were put back into the abdominal cavity. The abdominal cavity was then washed with physiological saline twice and closed by a two-layer silk suture. The postoperative status of mice was monitored every day. 3 weeks after injection, tumor-bearing mice were euthanized by cervical dislocation. The liver without macroscopic metastases was perfused with 20 mL PBS and collected for intrahepatic lymphocytes isolation (Figure 3).

3.2.5 Isolation of intrahepatic lymphocytes

Isolation of intrahepatic lymphocytes was carried out by the mechanical method. Firstly, using the plunger of the 2-mL syringe, the liver was squeezed through a 40 µM strainer. The strainer and the plunger were washed with PBS. The suspension was centrifuged at 300 g at room temperature (RT). Secondly, the supernatant was discarded and the pellet was resuspended in 45% Percoll solution. Liver cells were then loaded on the layer of 70% Percoll solution. The tube was centrifuged at 800 g for 20 min at RT without brake. Thirdly, intrahepatic lymphocytes were aspirated from the Percoll interface and washed twice with PBS. Cells was resuspended in PBS and counted for flow cytometric analysis (Figure 3).

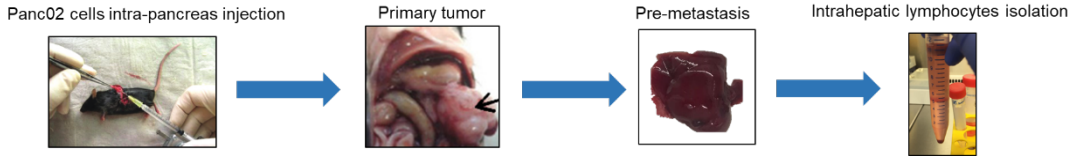

Figure 3. Establishment of orthotopic PDAC mouse models and isolation of intrahepatic lymphocytes.

3.2.6 Exosomes isolation

For exosomes preparation from cell culture supernatants, cells with a confluency of 70-80% were washed with DPBS for 3 times and were cultured in medium supplemented with 10% exosomes-free FBS for additional 24 hours. Exosomes were isolated by a differential centrifugation and ultracentrifugation method. In brief, supernatants were centrifuged at 300 g for 10 min and 2,000 g for 10 min at 4 °C to remove dead cells and cell debris. The supernatants were transferred into new tubes and centrifuged at 10,000 g for 30 min at 4 °C to remove large vesicles. The supernatants were then transferred into ultracentrifugation tubes and ultracentrifuged at 100,000 g for 70 min at 4 °C (Beckman Coulter, Optima^{TM} L-90K). After first round of ultracentrifugation, the supernanant was discarded. The pellet was resuspended in PBS and ultracentrifuged again at 100,000 g for 70 min at 4 °C. Exosomes were resuspended in 100 µL of PBS and stored in the -80°C freezer for future use.

Serum exosomes were isolated by a precipitation method using ExoQuick (System Biosciences) according to the manufacturer's instructions. In brief, serum samples were centrifuged at 3000 g for 15 min at RT to remove remaining blood cells and cell debris. After centrifugation, 250 μL of serum was put into a new tube and mixed with 63 μL of ExoQuick Exosome Precipitation Solution. The mixture was incubated at 4°C for 30 min and then centrifuged at 1500 g at 4°C for 30 min. The supernatant was aspirated. The tube was centrifuged at 1500 g for additional 5 min to remove the residual ExoQuick solution. The pellet was resuspended completely in 100 μL of PBS. Serum exosomes were stored in the -80°C freezer for future use.

3.2.7 Nanoparticle tracking analysis

The size distribution of pancreatic cancer-derived exosomes was examined by nanoparticle tracking analysis (NTA). Briefly, background measurements were performed with filtered PBS, which revealed the absence of any kinds of particles. Exosomes were diluted 1:1,000 with PBS. After sample loading, five repeated measurements were recorded and then analyzed using a Nanosight NS300 with the NTA 3.0 software (Malvern Instruments).

3.2.8 Transmission electron microscopy

The morphology of pancreatic cancer-derived exosomes was assessed by transmission electron microscopy (TEM). In brief, exosomes were put onto formvar-carbon-coated electron microscopy grids for 10 min in a wet chamber. After brief blotting the grid edge with filter paper, the grids were placed on drops of 2% aqueous uranyl acetate for 1 min, removed, blotted again, and placed on H_2O drops for 1 min, removed, and blotted at the edge. After 24 h of air drying, the grids were inspected using a transmission electron microscope (Zeiss EM 912 Omega at 100 kV).

3.2.9 Western blot

Cells and exosomes were lysed using complete lysis M buffer supplemented with phosphatase inhibitor cocktail (Roche). Protein samples were centrifuged at 14,000 g for 15 min at 4°C. After centrifugation, the supernatants were transferred into new tubes

and stored in the -80°C freezer. Protein concentrations were measured by BCA Protein Assay (Thermo Fisher Scientific). Protein Samples were prepared in Pierce™ LDS Sample Buffer (Thermo Fisher Scientific), boiled for 10 min at 70°C. 10-15 µg of lysates was loaded and run in SDS polyacrylamide gels. Gels were then transferred onto PVDF membranes. The membranes were blocked in Blocking Buffer at RT for 1 hour and incubated with specific primary antibodies (as detailed in materials 3.1.6.3) at 4°C overnight. Blots were washed 3 × 5 min in PBST. Incubation with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies were performed at RT for 1 hour. Blots were again washed in TBST 3 × 5 min. Proteins were detected via chemiluminescence with SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific) using Intas ChemoStar ECL Imager (Intas Science Imaging).

3.2.10 Flow cytometry of exosomes

Pancreatic cancer-derived exosomes were incubated with and polybead carboxylate microspheres (Polyscience) in PBS overnight at 4 °C. Samples were blocked with 2% BSA in PBS and centrifuged at 350 g for 2 min. The supernatants were discarded. The pellet was washed with PBS twice and resuspended in PBS. Exosomes were incubated with the anti-CD63 antibody at 4 °C for 20 min in the dark. The samples were analyzed by CytoFlex (Beckman Coulter).

3.2.11 Flow cytometry of cells

For surface staining, cells were incubated with Human TruStain FcX™ (Biolegend) on ice for 10 min. Then conjugated fluorescent antibodies were added and incubated on ice for 20 min in the dark. After incubation, cells were washed in PBS twice and then analyzed by flow cytometry. For intracellular staining, surface antigen staining was performed as above. Afterwards, cells were washed and fixed in fixation buffer (Biolegend) for 20 min at RT. Cells were washed and permeabilized with permeabilization wash buffer (Biolegend). Cells were incubated with appropriate conjugated fluorescent antibodies in the dark for 20 min at RT. After incubation, cells were washed with permeabilization wash buffer twice and analyzed by flow cytometry. For each sample, 30,000 to 50,000 events were acquired using CytoFlex (Beckman

Coulter). Data were analyzed using FlowJo software.

3.2.12 Mass spectrometry of exosomes

50 µg of pancreatic cancer-derived exosomes were used for mass spectrometry analyses. First, exosomes were denatured using 8 mol/L urea with Protease inhibitor cocktail. Samples were centrifuged for 15 min at 17,000g to remove debris. The protein concentration was determined using the Direct Detect® Spectrometer. Then 50 µg per sample was transferred into a new 1.5 mL tube. Samples were reduced using 5 mM DTT at 25 °C for 1 hour and incubated with 40 mM Chloroacetamide in the dark for 30 min. This was followed by proteolytic digestion with Lysyl Endopeptidase (Lys-C) at an enzyme:substrate ratio of 1:75 at 4 °C for 4 hours. The samples were diluted with 50 mM TEAB to achieve a final concentration of Urea ≤ 2M. A subsequent digestion with trypsin at an enzyme:substrate ratio of 1:75 and incubation at 25°C overnight was performed. The digestion was terminated with formic acid at a final concentration of 1%. After digestion, the peptide mixtures were desalted using in-house made StageTip per sample (containing 2 layers of SDB-RPS discs). StageTips were equilibrated as follows: 20 µL of Methanol, centrifugation at 2,600 rpm for 1 min; 20 µL of 0.1% formic acid in 80% Acetonitrile, centrifugation at 2,600 rpm for 1 min; 20 µL of 0.1% formic acid in water, centrifugation at 2,600 rpm for 1.5 min; 20 µL of 0.1% formic acid in water, centrifugation at 2,600 rpm for 2 min. The samples (acidified with formic acid) were centrifuged at full speed for 5 min and then loaded onto the equilibrated StageTips. After centrifugation at 2,600 rpm for 5 min, the StageTips were washed according to the following protocol: 30 µL of 0.1% formic acid in water, centrifugation at 2,600 rpm for 3 min; 30 µL of 0.1% formic acid in 80% Acetonitrile, centrifugation at 2,600 rpm for 3 min. The last wash step was performed twice. Finally, the StageTips were dried completely with a syringe and kept at 4 °C until LC-MS Analysis. Nano LC-MS was performed using a gradient for 150 min and analyzed using the MAXQuant and Perseus software.

3.2.13 *In vitro* exosomes uptake assay

Pancreatic cancer cells-derived exosomes were isolated as described above. PKH67

Fluorescent Cell Linker kits (Merck) was used to label exosomes according to the manufacturer's instruction. In brief, after first round of ultracentrifugation, the supernatant was discarded and the pellet of exosomes was resuspended in 750 µL of Diluent C. 1 µL of PKH67 dye was dissolved in 250 µL of Diluent C. Exosomes and PKH67 dye were mixed gently and incubated at RT for 5 min. 9 ml of PBS with 1% BSA was added to bind excess PKH67 dye. The exosomes were ultracentrifuged at 100,000 g for 70 min at 4 °C and washed twice in PBS by ultracentrifugation. The PKH67-labeled exosomes were then resuspended in PBS. NK cells were incubated with PKH67-labeled exosomes for 24 h and put on polysine adhesion slides for 30 min at 37 °C. After fixation and permeabilization, NK cells were stained with DAPI. Uptake of PKH67-labeled exosomes by NK cells was visualized by confocal microscopy.

3.2.14 *In vivo* distribution of exosomes

To study the *in vivo* distribution of pancreatic cancer-derived exosomes, exosomes were fluorescently labeled as described above. Animal experiments were conducted according to protocols approved by the responsible national and local authority (81-02.04.2018.A139, LANUV NRW, approved on 20th September 2018). PKH67-labeled exosomes were administered into the tail vein of two healthy 4–6-week-old NSG mice. One NSG mouse was injected with PBS as a negative control. Twenty four hours after injection, mice were euthanized. Organs were dissected and embedded in Tissue-Tek O.C.T.TM. Then samples were frozen and stored at −80 °C. For immunofluorescence, 10 µm of O.C.T.TM tissue cryosections were stained with DAPI. The distribution of PKH67-labeled exosomes was analyzed by confocal microscopy.

3.2.15 *In vitro* NK cell cytotoxicity assay

L3.6pl cells (2×10^5) were plated with NK cells (effector:target=5:1)in a 6-well plate. All wells contained 100 IU/mL rhIL-2 with 50% of NK cell medium and 50% of L3.6pl culture medium. After co-culture for 24 hours, plates were washed with DPBS for three times. Adherent cells were harvested and counted for subsequent experiments.

3.2.16 Sphere formation assay

L3.6pl cells were seeded as single cell suspension at a concentration of 2000 cells/well in 6-well ultra-low attachment plates (Corning, USA) in tumor sphere formation assay medium as described above. After 7 days, spheres were counted under microscopy. Spheres were collected for mRNA extraction and flow cytometric analysis.

3.2.17 RNA isolation, cDNA synthesis and qRT-PCR

Total RNA was isolated from cultured cells according to the manufacturer's instructions using the RNeasy Mini Kit (QIAGEN). RNA was reverse transcribed according to the manufacturer's protocol using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The synthesized cDNA was then assessed for gene expression using the Fast SYBR green master mix (Invitrogen) with QuantStudio 7 flex (Applied Biosystems).

3.2.18 Human studies

All human tissue and blood samples were collected ethically and their research use was according to the terms of the informed consents (BIOMASOTA, ID: 13-091, approval in May 2016). Human peripheral blood samples were acquired from healthy subjects and PDAC patients at University Hospital of Cologne between October 2016 and December 2018. All patients with PDAC were pathologically confirmed. Blood was obtained and centrifuged at 2,000 rpm for 10 min and 4,000 rpm for 10 min at RT. Serum samples were aliquoted and store in the -80°C freezer. Serum exosomes were isolated as described above in the "4.2.5 exosomes isolation" part. Exosomal TGF-β1 levels in serum were determined using TGF beta-1 Human/Mouse Uncoated ELISA Kit (eBiosciences) and analyzed with an ELISA microplate reader at 450 nm.

3.2.19 Statistical analysis

Differences between two groups were tested by two-tailed Student's t test. All statistical analyses were made using GraphPad Prism 8. Data were considered statistically significant when p value was smaller than 0.05.

IV. RESULTS

4.1 Characterization of pancreatic cancer-derived exosomes

Exosomes were isolated from cell culture supernatants of a highly metastatic pancreatic cancer cell line L3.6pl and a PDAC patient derived primary cancer cell line TBO368 by differential centrifugation and ultracentrifugation to exclude dead cells, large debris, and microvesicles (Figure. 4a). In order to examine the morphology and measure the size of pancreatic cancer-derived exosomes, we used transmission electron microscopy (TEM) and nanoparticle analysis (NTA). The image of TEM showed that pancreatic cancer-derived exosomes displayed features of membrane vesicles (Fig. 4b). The result of NTA demonstrated that most of pancreatic cancer-derived exosomes had a diameter around 110 nm (Fig. 4c). Exosomes were further characterized by their expression of CD9, CD63, CD81, ALIX, Flotillin-1, TSG101 and Rab5, which are commonly used markers for exosomes (Fig. 4d & e). Intriguingly, we also detected mutant KRAS (G12D) in L3.6pl-derived exosomes, which was consistent with that in genomic DNA (Fig. 4f).

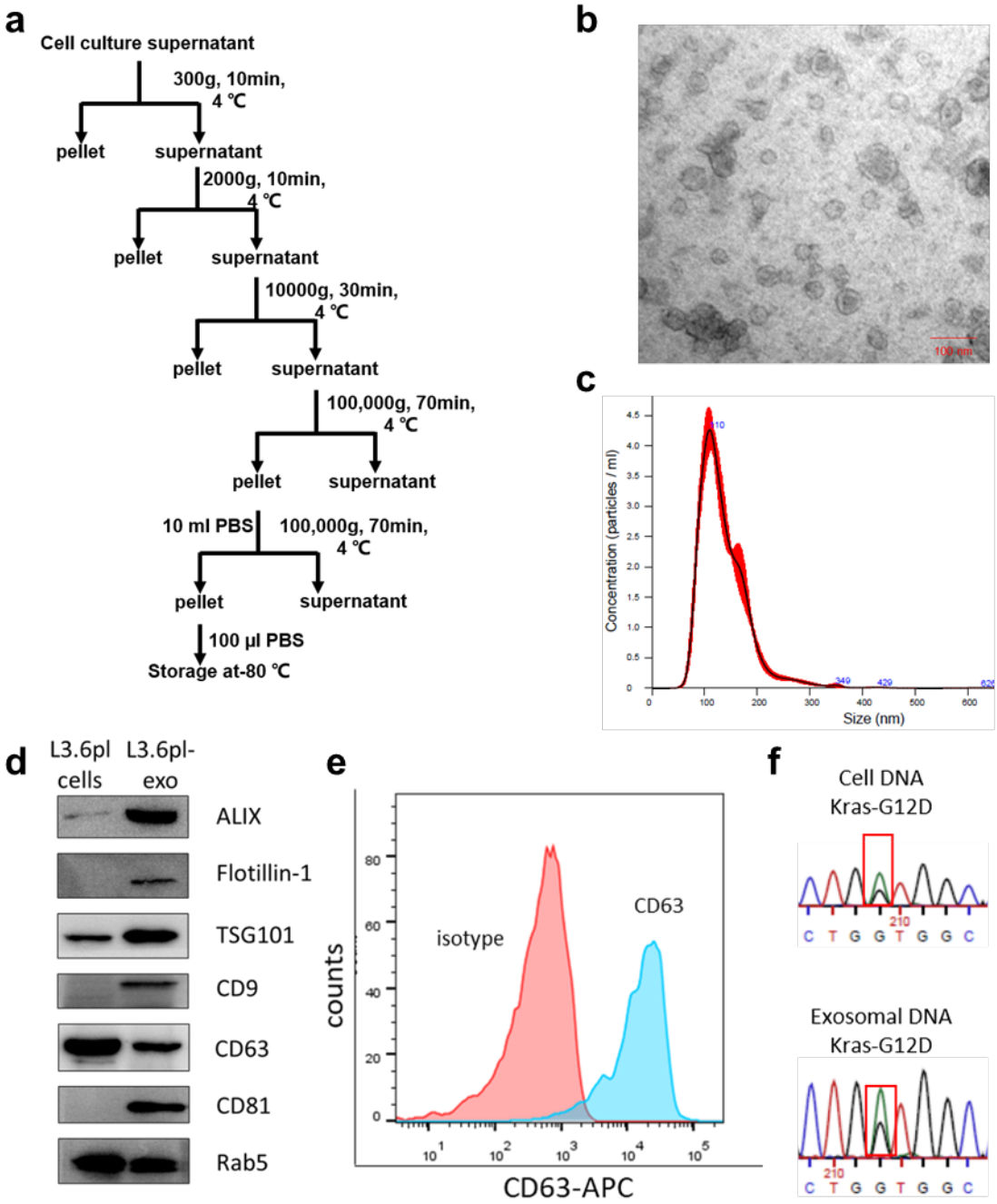

Figure 4. Characterization of pancreatic cancer cell-derived exosomes. a) Exosomes were isolated by differential centrifugation and ultracentrifugation. b) The representative image of pancreatic cancer-derived exosomes by TEM. Scale bar, 100 nm. c) The size of pancreatic cancer-derived exosomes was determined by NTA. The size range was 136.1 ± 47.3 nm. d) The expression of exosomal markers ALIX, Flotillin-1, TSG101, CD9, CD63, CD81and Rab5 for L3.6pl-derived exosomes and parental cells was determined by Western blotting. e) The expression of CD63 on

L3.6pl-derived exosomes coupled to carboxylatex beads was analyzed by flow cytometry. f) Mutant KRAS (G12D) was detected both in both genomic DNA and L3.6pl-derived exosomes.

4.2 Comprehensive proteomic analysis of pancreatic cancer-derived exosomes

The proteomic profile of pancreatic cancer-derived exosomes was analyzed by mass spectrometry. More than 2,600 proteins were detected in both samples. A significant overlap was observed in L3.6pl-derived exosomes and TBO368-derived exosomes. (Fig. 5a). 88 of the top 100 most frequently identified exosomal proteins, according to the Exocarta database (http://www.exocarta.org), were detected in pancreatic cancer-derived exosomes. The enrichment of exosomal markers verified the purity of exosomes (Fig. 5b).

To investigate the cellular component, molecular function and biological process of proteins in pancreatic cancer-derived exosomes, GO analysis was performed using the Gene Ontology Resource (http://geneontology.org/). Proteins were categorized according to their ontology as determined from their GO annotation terms. Based on the cellular component, around 40% of all the identified proteins were annotated to extracellular exosomes (Fig. 5c). The molecular function revealed the enrichment of proteins related to translation regulator activity (GO:0045182), transcription regulator activity (GO:0140110), molecular transducer activity (GO:0060089), binding (GO:0005488), structural molecule activity (GO:0005198), molecular function regulator (GO:0098772), catalytic activity (GO:0003824), transporter activity (GO:0005215) (Fig. 5d). The biologic process revealed the proteins in pancreatic cancer-derived exosomes were involved in cellular component organization or biogenesis (GO:0071840), cellular process (GO:0009987), biological phase (GO:0044848), localization (GO:0051179), reproduction (GO:0000003), biological regulation (GO:0065007), response to stimulus (GO:0050896), developmental process (GO:0032502), multicellular organismal process (GO:0032501), biological adhesion (GO:0022610), metabolic process (GO:0008152), cell proliferation (GO:0008283),

immune system process (GO:0002376) (Fig. 5e).

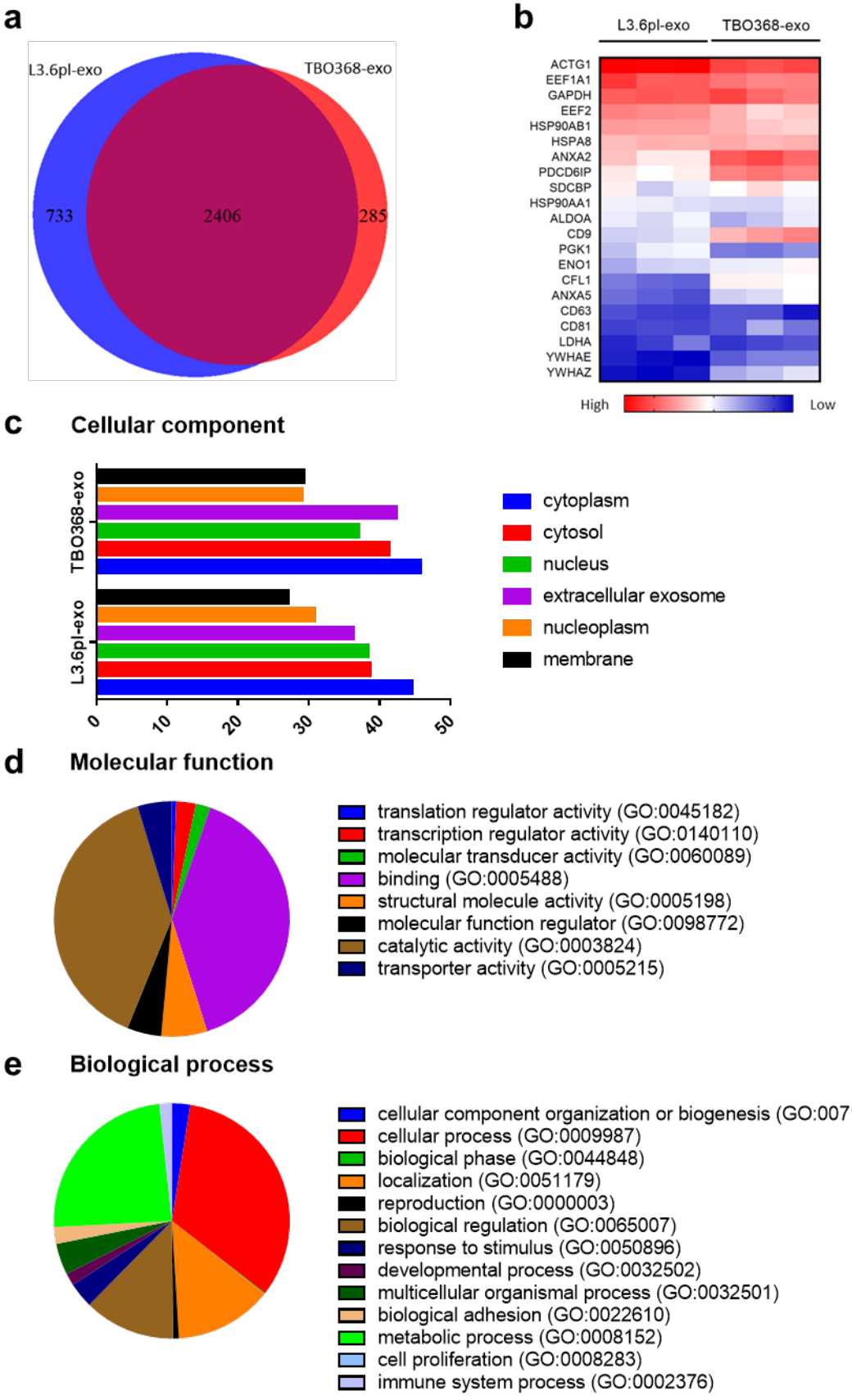

Figure 5. Proteomic analysis of pancreatic cancer-derived exosomes. a) Proteomic analysis identified 3,139 proteins in L3.6pl-derived exosomes and 2,691 proteins in TBO368-derived exosomes. The Venn diagram showed an overlap of 2,406 proteins in both samples. b) Heatmap showed enrichment of typical exosomal markers in L3.6pl-derived exosomes and TBO368-derived exosomes. c) The cellular component of proteins in TBO368-derived exosomes and L3.6pl-derived exosomes. d) The molecular function of identified proteins in pancreatic cancer-derived exosomes. e) The biological process of identified proteins in pancreatic cancer-derived exosomes.

4.3 Pancreatic cancer-derived exosomes carry adhesion molecules

To evaluate the role of pancreatic cancer-derived exosomes in the pre-metastatic niche, GO analysis revealed abundant cellular adhesion proteins existed in pancreatic cancer-derived exosomes, particularly the integrins, such as ITGA1, ITGA2, ITGA3, ITGA6, ITGAV, ITGB1, ITGB4, ITGB5, ITGB6 and ITGB8(Fig. 6a & b). We detected the expression of Integrin alpha V (ITGAV) in L3.6pl-derived exosomes by Western blotting (Fig. 6c). To track *in vivo* distribution of pancreatic cancer-derived exosomes, we further injected PKH67-labelled L3.6pl-derived exosomes intravenously into the NSG mice. 24 hours after injection, PKH67-labelled exosomes were detected by immunofluorescence in the cryosection of mouse liver tissue, which indicated that pancreatic cancer-derived exosomes reached the liver (Fig. 6d).

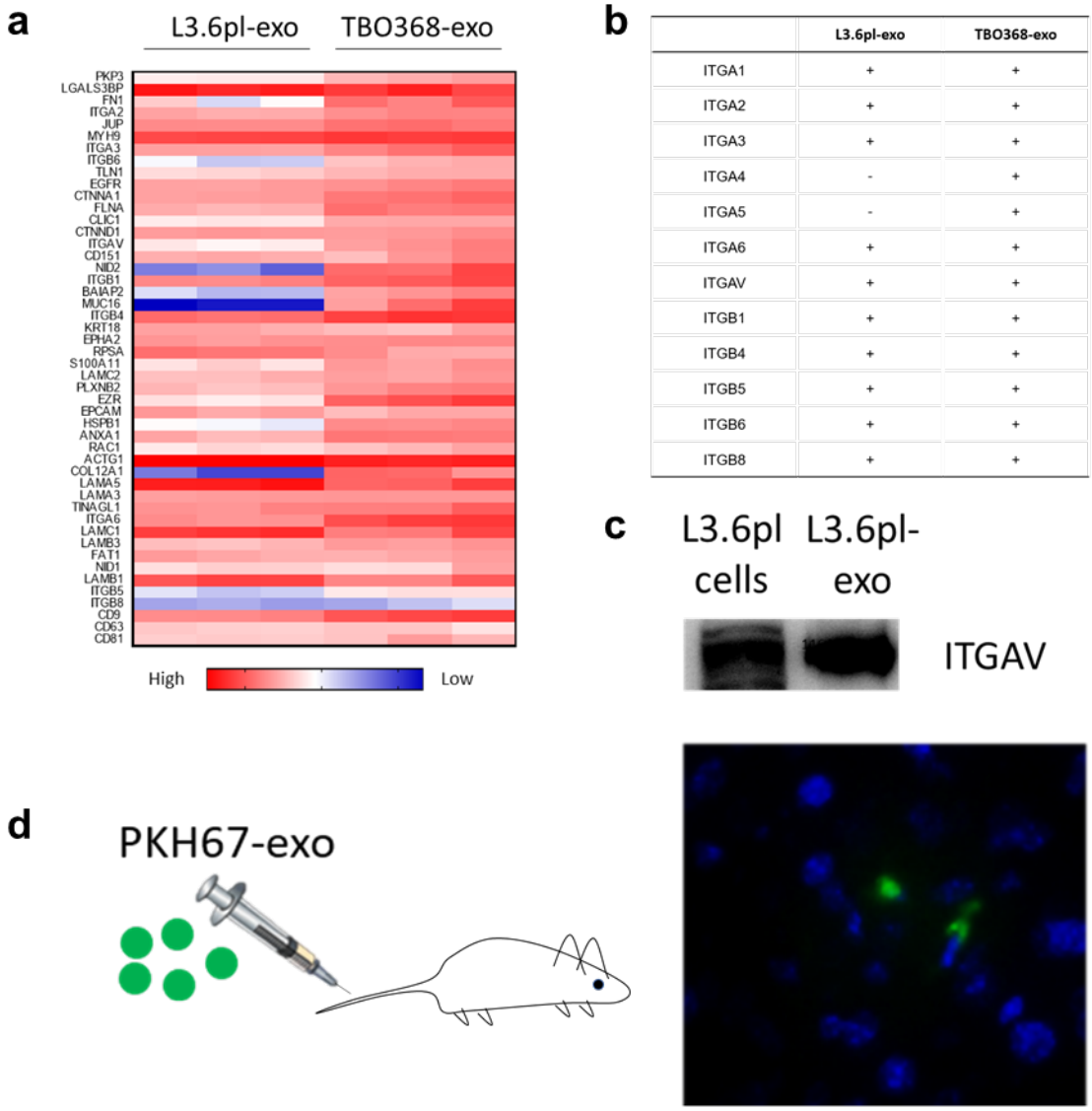

Figure 6. Pancreatic cancer-derived exosomes carry adhesion molecules. a) Heatmap of adhesion molecules in L3.6pl-derived exosomes and TBO368-derived exosomes, exosomal markers CD9, CD63, CD81 as internal references. b) Integrins in L3.6pl-derived exosomes and TBO368-derived exosomes. c) Western blot analysis of ITGAV in L3.6pl-derived exosomes. d) Analysis of liver injected with PKH67-labeled L3.6pl-derived exosomes (green) by confocal microscopy. Nuclei were stained with DAPI (blue).

4.4 Pancreatic cancer-derived exosomes carry immune regulatory factors

To investigate the role of tumor-derived exosomes in immune regulation, we first analyzed the expression pattern of immune regulatory factors in paired PDAC tumor

tissues and adjacent non-tumor tissues based on the GSE28735 dataset (n=45). Compared to non-tumor tissues (N), a variety of factors like TGF-β1, TGF-β2, HMGB1, PVR, Nectin-2, Galectin-9, PD-L1, PD-L2 and MICA/MICB were significantly higher in the tumor tissue (T) (Fig. 7a). Interestingly, enrichment of some molecules, including TGF-β1, Nectin-2 and PVR, was demonstrated in pancreatic cancer-derived exosomes by Western blotting (Fig. 7b). TGFbRI and TGFbRII (TGF-β1 receptors), DNAM-1, TIGIT and CD96 (Nectin-2 and PVR receptors) are present on NK cells. These results support the hypothesis that pancreatic cancer-derived exosomes potentially modulate NK cell function.

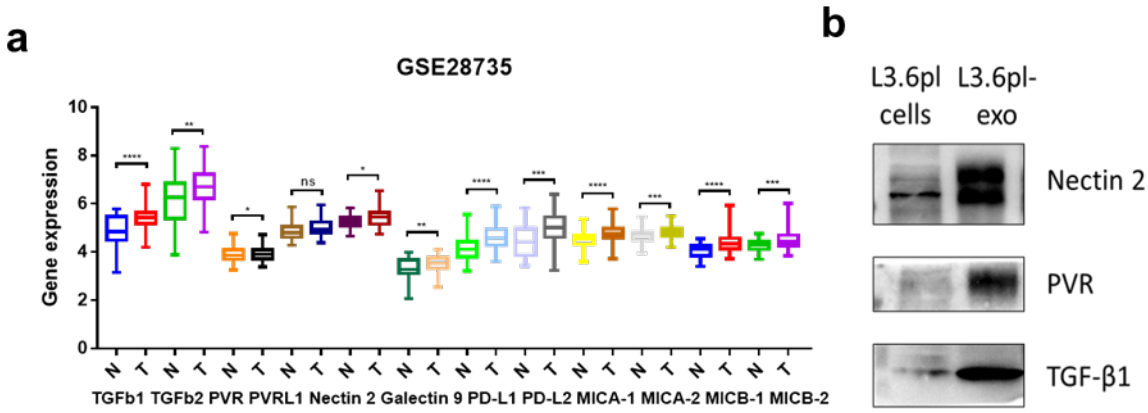

Figure 7. Immune regulatory factors in PDAC and pancreatic cancer-derived exosomes. a) Relative mRNA expression of representative immune regulatory factors in tumor tissues (T) and non-tumor tissues (N) in pancreatic cancer from GSE28735 dataset, n = 45. b) The expression of Nectin-2, PVR and TGF-β1 was determined by Western blotting in L3.6pl-derived exosomes and L3.6pl cells. ns, no significant difference, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, **** $P < 0.0001$ by Student's *t* test.

4.5 Intrahepatic lymphocytes in hepatic pre-metastatic niche of PDAC

It is difficult to explore the hepatic pre-metastatic niche in patients with PDAC due to lack of specimen. Thus, we established the orthotopic PDAC mouse models by injecting Panc02 cells into the pancreas. Intrahepatic lymphocytes were isolated from the hepatic pre-metastatic niche, which was examined by microscopy to make sure no

evidence of macro-metastasis or micro-metastasis. We found that there was no significant difference of the percentage of T cells, B cells or group 1 innate lymphoid cells (ILCs) in $CD45^+$ cells between the PDAC-bearing mice and healthy mice (Figure. 8b). We then analyzed the percentage of NK cells in group 1 ILCs. Surprisingly, a significant decrease of the proportion of NK cells in group 1 ILCs was observed in PDAC group (Figure. 8c). This result suggested impaired immune surveillance of NK cells in the hepatic pre-metastatic niche of PDAC.

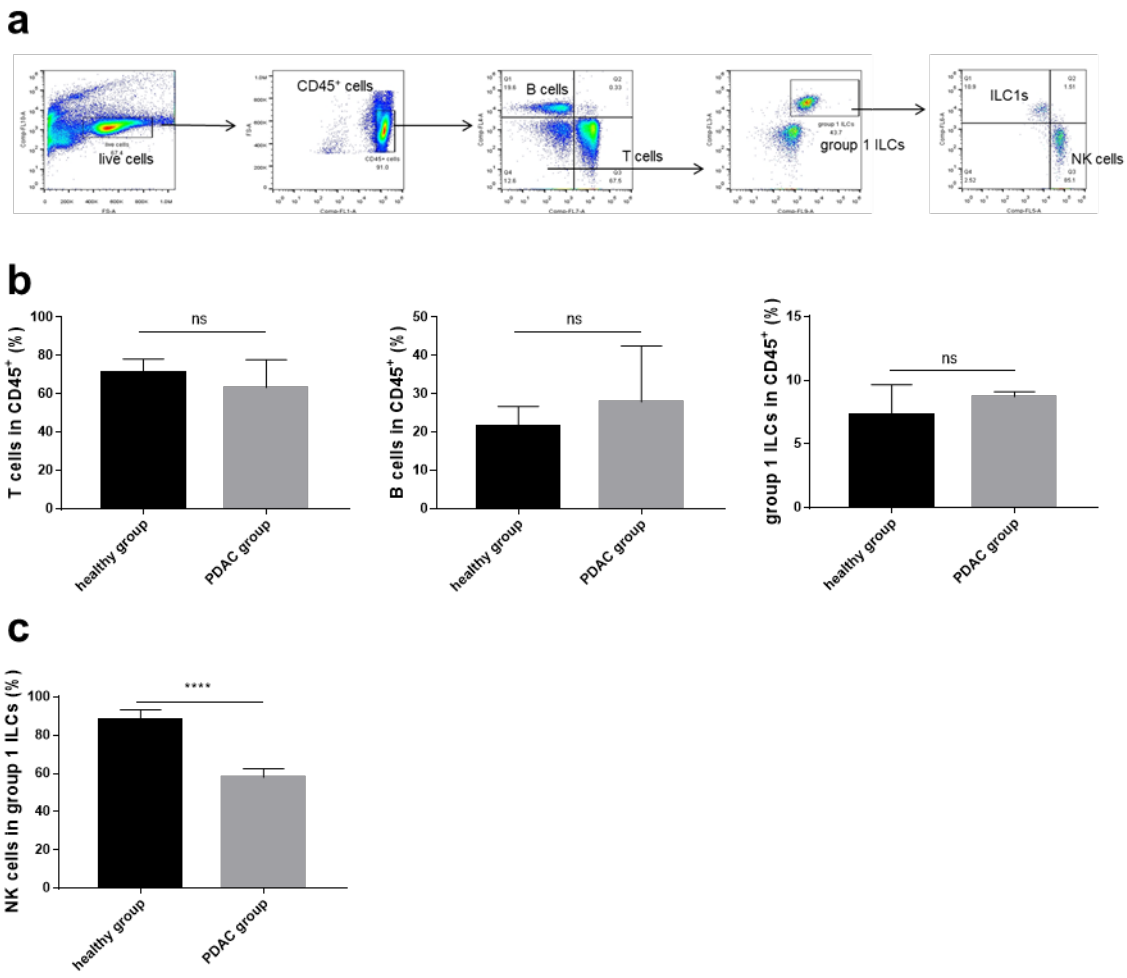

Figure 8. Intrahepatic lymphocytes in the hepatic pre-metastatic niche of PDAC. a) Flow cytometry gating strategy for T cells, B cells, group 1 ILCs, NK cells and ILC1s. T cells: live $CD45^+$ $CD3^+$, B cells: live $CD45^+$ $CD3^-$ $CD19^+$, Group 1 ILCs: live $CD45^+$ $CD3^-$ $CD19^-$ $NK1.1^+$ $NKp46^+$, NK cells: live $CD45^+$ $CD3^-$ $CD19^-$ $NK1.1^+$ $NKp46^+$ $CD49a^-$ $CD49b^+$, ILC1s: live $CD45^+$ $CD3^-$ $CD19^-$ $NK1.1^+$ $NKp46^+$ $CD49a^+$ $CD49b^-$. b)

The percentage of T cells, B cells and group 1 ILCs in intrahepatic $CD45^+$ cells from PDAC-bearing mice and healthy controls. c) The percentage of NK cells in intrahepatic group 1 ILCs from PDAC-bearing mice and healthy controls. Data are means ± SD of four mice per group. ns, no statistically significant difference, ****$P < 0.0001$ by Student's *t* test.

4.6 Pancreatic cancer-derived exosomes inhibit NK cell function

Subsequently, we determined whether NK cell could take up pancreatic cancer-derived exosomes. To address this issue, L3.6pl-derived exosomes were stained with PKH67 (green). PKH67-labelled exosomes were incubated with NK cells. After 24 hours, we observed PKH67-lablled exosomes were present on the plasma membrane and in the cytoplasma of NK cells (Fig. 9a). This result indicated that pancreatic cancer-derived exosomes could be incorporated by NK cells, suggesting their potential role in the regulation of NK cell function. Thus, we examined the effects of pancreatic cancer-derived exosomes on NK cell.

NKG2D is one of the most important activating receptors on NK cells and the expression level of NKG2D correlates positively with their anti-tumor ability[104]. We co-cultured NK cells with L3.6pl-derived exosomes or PBS for 24 hours. After co-culture, the expression of NKG2D in NK cells was significantly downregulated (Fig. 9b). CD107a is a functional marker for NK cells[105]. TNF-α and IFN-γ are two main cytokines produced by activated NK cells[106]. To measure the amount of CD107a, TNF-α and IFN-γ, NK cells pre-treated with L3.6pl-derived exosomes or PBS were co-cultured with L3.6pl cells at an effector:target cell ratio of 1:1 for 5 hours. L3.6pl-derived exosomes resulted in a significant decrease of CD107a, TNF-α and IFN-γ in NK cells (Fig. 9c). Nutrient uptake and glucose metabolism are essential for NK cell functionality[66]. CD71 (transferrin receptor), CD98 (large neutral amino acid transporter), and 2-NBDG incorporation ability are three commonly used metabolic parameters in NK cells[107]. We found that L3.6pl-derived exosomes significantly reduced the expression of CD71 and CD98 in NK cells. In addition, L3.6pl-derived

exosomes impaired the glucose uptake ability of NK cells (Fig. 9d).

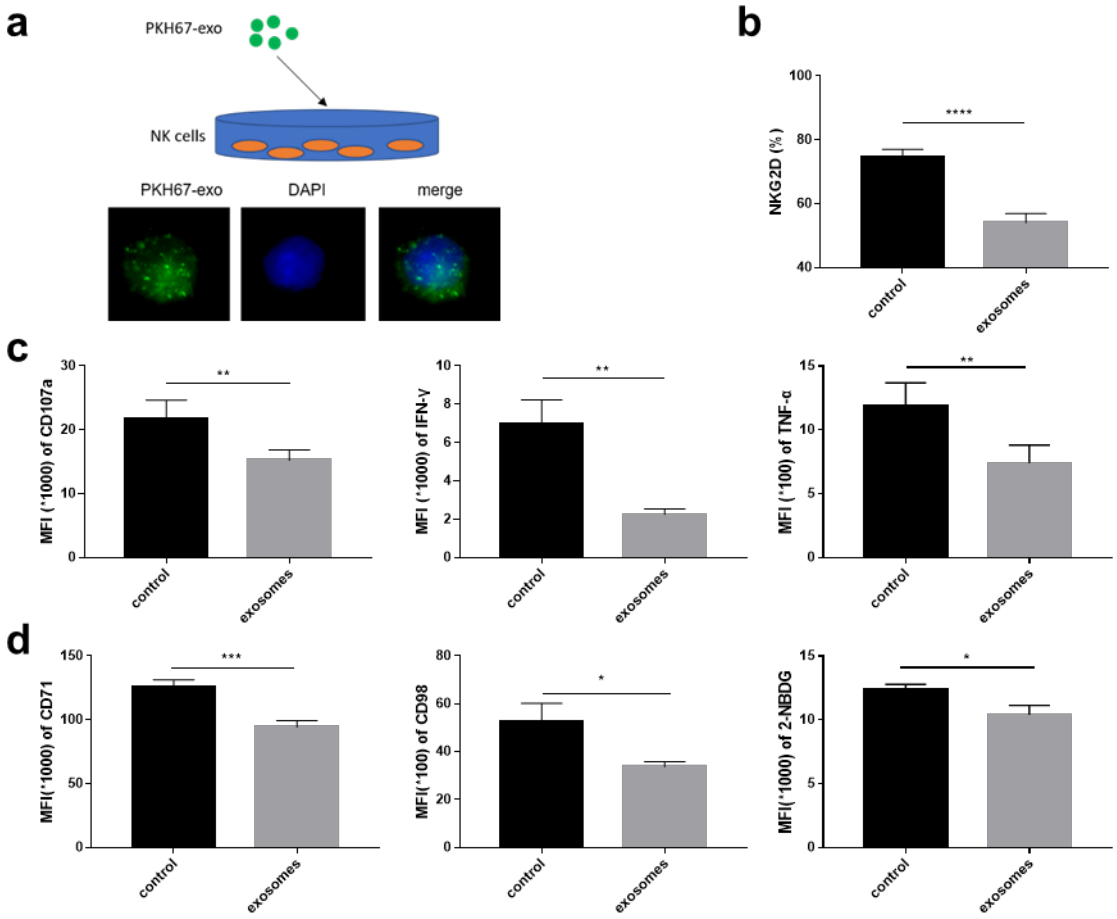

Figure 9. Pancreatic cancer-derived exosomes impair NK cell function. a) Analysis of pancreatic cancer-derived exosomes uptake by NK cells using confocal microscopy. L3.6pl-derived exosomes were stained with PKH67 (green) and incubated with NK cells for 24 hours. The nucleus was labeled with DAPI (blue). b) NK cells were treated with PBS or L3.6pl-derived exosomes for 24 hours. The percentage of NKG2D positive NK cells were analyzed by flow cytometry. c) NK cells pre-treated with PBS or L3.6pl-derived exosomes were co-cultured with L3.6pl cells at a 1:1 ratio for 5 hours. The MFI of CD107a (left), IFN-γ (middle) and TNF-α (right) in NK cells was analyzed by flow cytometry. d) NK cells were treated with PBS or L3.6pl-derived exosomes for 24 hours. NK cells were then analyzed by flow cytometry to determine the MFI of CD71 (left), and CD98 (middle), 2-NBDG incorporation (right). Data are means ± SD of four experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, **** $P < 0.0001$ by Student's *t* test.

4.7 Pancreatic cancer-derived exosomes impair NK cell cytotoxicity

It has been reported NK cells have the capacity to kill CSCs[108]. We next examined whether pancreatic cancer-derived exosomes impaired NK cell cytotoxicity against pancreatic CSCs. After enrichment of CSCs using sphere formation assay, we found higher mRNA expression levels of NKG2D ligands, MICB and ULBP2 in the CSCs population (Figure. 10a). Flow cytometric analysis confirmed a higher MICA/MICB expression in spheres than that in adherent cells (Figure. 10b). This indicated that NK cells might prefer to recognize and eliminated pancreatic CSCs. NK cells were then pre-treated withL3.6pl-derived exosomes in the presence of IL-2 (100 U/ml) for 24 hours. Then we co-cultured L3.6pl cells with untreated or L3.6pl-derived exosomes pre-treated NK cells. After 24-hour killing, floating cells were washed away and adherent cells were trypsinized for sphere formation assay (Figure. 10c). we found NK cells pre-treated with L3.6pl-derived exosomes showed decreased cytotoxicity against pancreatic CSCs (Figure. 10d).

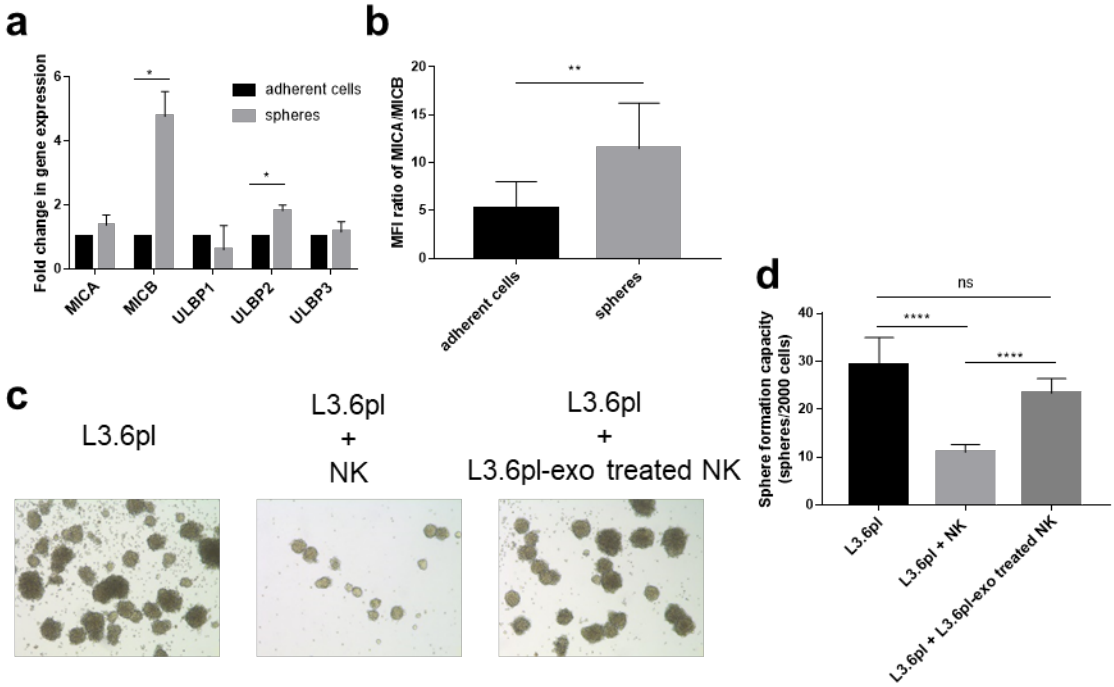

Figure 10. Pancreatic cancer-derived exosomes suppress NK cell cytotoxicity against CSCs. a) Gene expression of NKG2D ligands in adherent cells and spheres using qRT-PCR. Data are normalized to GAPDH and presented as fold change in

comparison with genes in adherent cells. b) The MFI of MICA/MICB in adherent cells and spheres was determined by flow cytometry. c) Representative images of tumor spheres without NK cell killing(left), tumor spheres after untreated NK cell killing (middle), and tumor spheres after L3.6pl-derived exosomes pre-treated NK cell killing (right). d) The number of tumor spheres without NK cell killing, tumor spheres after untreated NK cell killing, and tumor spheres after L3.6pl-derived exosomes pre-treated NK cell killing. Data are means ± SD of four experiments. *P < 0.05, **P < 0.01, **** P < 0.0001 by Student's t test.

4.8 Pancreatic cancer-derived exosomes phosphorylate Smad2/3 in NK cells

As a major immunosuppressive cytokine, TGF-β1 inhibits the activation and function of NK cells through the TGFβ-Smad2/3 signaling pathway[109]. In our experiments, we observed that TGF-β1 attenuated the expression of NKG2D, CD107a, IFN-γ, CD71, CD98 and 2-NBDG incorporation ability of NK cells (Fig. 11a, b & c). As shown above, pancreatic cancer-derived exosomes contained TGF-β1. Therefore, we investigated whether pancreatic cancer-derived exosomes could activate the TGFβ-Smad2/3 signaling pathway in NK cells. After incubation with TGF-β1 or L3.6pl-derived exosomes, the phosphorylation level of Smad2/3 in NK cells was significantly elevated. However, in the presence of SB-431542 (an inhibitor of TGFβRI), the phosphorylation of Smad2/3 was reversed and returned to the original baseline level (Fig. 11d). These findings suggest that pancreatic cancer-derived exosomes deliver TGF-β1 to NK cells, induce Smad2/3 phosphorylation, and ultimately result in NK cell dysfunction.

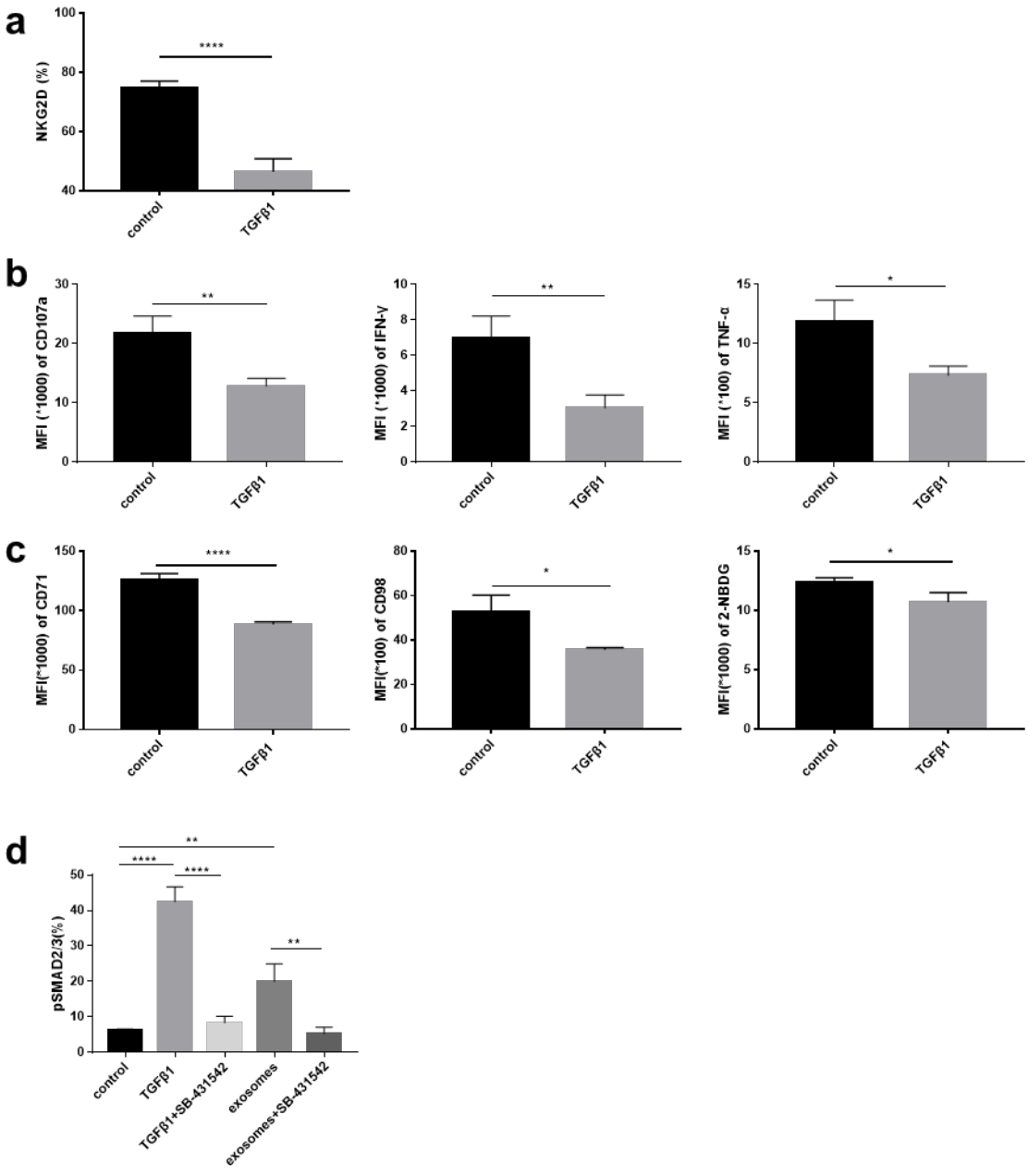

Figure 11. Pancreatic cancer-derived exosomes inhibit NK cell function through the TGFβ-Smad2/3 signalling pathway. a) NK cells were treated with PBS or TGF-β1 for 24 hours. The percentage of NKG2D positive NK cells were analyzed by flow cytometry. b) NK cells pre-treated with PBS or TGF-β1 were co-cultured with L3.6pl cells at a 1:1 ratio for 5 hours. The MFI of CD107a (left), IFN-γ (middle) and TNF-α (right) in NK cells was analyzed by flow cytometry. c) NK cells were treated with PBS or TGF-β1 for 24 hours. NK cells were then analyzed by flow cytometry to determine the MFI of CD71 (left), and CD98 (middle), 2-NBDG incorporation (right). d) After

co-culture with TGF-β1 or L3.6pl-derived exosomes in the presence or absence of SB-431542, the phosphorylation level of SMAD2/3 in NK cells was measured by flow cytometry. Data are means ± SD of four experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, **** $P < 0.0001$ by Student's *t* test.

4.9 Increased exosomal TGF-β1 in serum of patients with PDAC

The clinicopathological characteristics of patients with PDAC are listed in Table 2. The mean age of the patients was 66.1 yrs, and they were predominantly male. All the patients donated blood at the time point of diagnosis prior to any treatment (n=30). At diagnosis, 53.3% of patients presented with a tumor stage T1 and T2, and 46.7% presented with T3 or T4. 77.7 % of patients had a positive lymph node status. 3 patients had distant metastases (M1). 56.7% of the patients were UICC I or II and 43.3% were UICC III or IV. 19 healthy individuals were included in this study as control (n=19). TGF-β1 was overexpressed in PDAC (Fig. 12a). The amount of TGF-β1 in serum exosomes was determined by ELISA. The concentration of TGF-β1 per gram of exosomes was calculated. The level of exosomal TGF-β1 in the patients with pancreatic cancer ranged from 0.20 to 0.88 ng/g. In the healthy donors, it ranged from 0.05 to 0.30 ng/g. Compared to healthy donors, TGF-β1 in serum exosomes was significantly elevated in patients with PDAC ($P<0.0001$) (Fig. 12b).

Table 2. The clinicopathological characteristics of patients with PDAC

Clinicopathological data	Patients (n=30)	
	n	%
Average age (years)	66.1	
<=60	9	30%
>60	21	70%
Gender		
Male	19	63.3%
female	11	36.7%
Tumor stage		
T1	4	13.3%
T2	12	40%
T3	11	36.7%
T4	3	10%
Nodal status		
N0	7	23.3%
N1	10	33.3%
N2	13	43.3%
Distant metastasis		
M0	27	90%
M1	3	10%
UICC stage		
I	6	20%
II	11	36.7%
III	10	33.3%
IV	3	10%

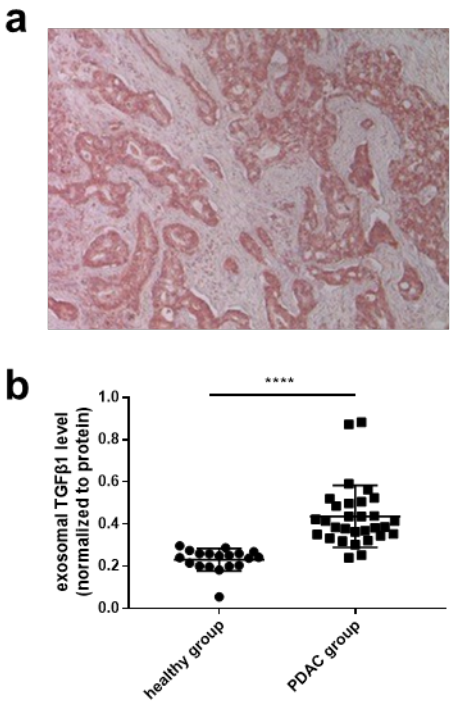

Figure 12. Elevated serum exosomal TGF-β1 in pancreatic cancer patients. a) The IHC result showed TGF-β1 overexpression in tumor tissue of PDAC. b) The amount of TGF-β1 per gram of serum exosomes in healthy control (n = 19) and PDAC group (n = 30).

V. DISCUSSION

Pancreatic ductal adenocarcinoma is one of the most lethal malignancies[1]. Metastasis accounts for a majority of cancer-related deaths in PDAC. Recently, the pre-metastatic niche has been proposed to elucidate the mechanisms of the organ-specific metastatic process in many cancer entities, such as melanoma, lung cancer and pancreatic cancer[28, 96, 110]. Over the past few decades, exosomes have attracted wide attention in early detection, diagnosis and treatment of cancer. Moreover, as a mediator of intercellular communication, exosomes released from tumor cells are found to interact with cells in distant organ sites, and finally induce a pre-metastatic niche for future metastasis[111]. Despite tremendous advances, the underlying cellular and molecular events involved in pre-metastatic niche formation of PDAC have yet to be determined.

In the present study, tumor-derived exosomes were isolated from two human pancreatic cancer cell lines, L3.6pl and TBO368 by differential centrifugation and ultracentrifugation to exclude dead cells, large debris, and microvesicles. Then the morphology and size distribution of exosomes were examined by TEM and NTA. Exosomal markers, including CD9, CD63, CD81, TSG101, Alix, Flotillin-1 and Rab5 were identified by Western blotting. Comprehensive proteomic analysis is expected to elucidate the potential impact of tumor-derived exosomes on pre-metastatic niche formation of PDAC. The proteomic profile of mouse PDAC cell line-derived exosomes has been analyzed by previous study. Yu Z *et al.* compared exosomes derived from Panc02 and Panc02-H7 cells using proteomic analyses. The differentially expressed proteins in Panc02-H7-derived exosomes were thought to enhance tumor growth, invasion and metastasis[112]. To the best of our knowledge, it is the first study to systematically analyze the protein content in human PDAC-derived exosomes. By mass spectrometry, more than 2,600 proteins were detected in both samples. We found that about 90% of the protein identified in L3.6pl-derived exosomes overlapped with those identified in TBO368-derived exosomes. GO analysis of identified proteins was

performed for cellular components, molecular functions and biologic processes.

GO-based category clustering of the molecular functions of protein contents in pancreatic cancer-derived exosomes suggested that there was a significant enrichment in localization and biological adhesion, which may facilitate the ability of exosomes to adhere to the surfaces of recipient cells, fuse with their membranes, and transfer exosomal components into the target cells to modulate their biological functions. The mechanisms of organ-specific homing and colonization of cancer cells are enormously complex. Cell adhesion to the extracellular matrix (ECM) determines the colonization of metastatic sites and facilitates the survival of circulating tumor cells in the new environment. Integrins can bind to fibronectin, vitronectin, laminin, and collagen in ECM, thereby enhancing tumor cell motility and invasion ability[113, 114]. Y Liu *et al*. reported that after either intravenous injection or intra-tumor injection, lung cancer-derived exosomes were detected in the lung and induced a lung pre-metastatic niche in mouse models[96]. GO analysis revealed that pancreatic cancer-derived exosomes exhibited abundant cellular adhesion molecules, especially integrins. Ayuko Hoshino *et al*. found that tumor-derived exosomal integrins determined organotropic metastasis. They reported tumor-derived exosomes carrying integrins α6β4 and α6β1 were responsible for lung metastasis, while exosomes carrying integrin αvβ5 were associated with liver metastasis[98]. Our findings showed that integrin αv and integrin β5 were abundant in pancreatic cancer-derived exosomes. After intravenous injection, PKH67-labeled pancreatic cancer-derived exosomes reached the liver of the NSG mouse. Therefore, we proposed that tumor-derived exosomes tended to enter the liver, delivered cargos to the recipient cells, and induced a pre-metastatic niche for future metastasis in PDAC.

A key feature of the pre-metastatic niche is immunosuppression[27]. Chen G *et al*. found that exosomal PD-L1 mediated immune evasion and could be used to predict efficacy of anti-PD-1 therapy in metastatic melanoma[115]. Chang-Sook Hong *et al*. reported that circulating exosomes containing immunosuppressive factors interfered

immune response in acute myeloid leukemia[116]. In our study, GO analysis revealed that identified proteins in pancreatic cancer-derived exosomes were involved in biological regulation and immune system process. By Western blotting, we found pancreatic-cancer derived exosomes displayed a variety of immune regulatory molecules, such as TGF-β1, Nectin-2 and PVR. Therefore, we speculated that pancreatic cancer-derived exosomes might be involved in the modulation of immune cell functions.

Most PDAC patients with liver metastases lose the opportunity for curative surgery[1]. Hence, it's difficult to collect liver samples from PDAC patients. Therefore, we explored the hepatic pre-metastatic niche in orthotopic PDAC mouse models. In mice, group 1 ILCs consist of NK cells and type 1 ILCs. The conversion of effector NK cells into type 1 ILCs was reported as a mechanism by which tumor cells escaped from immune surveillance[117]. Interestingly, we discovered that compared to healthy mice, the percentage of NK cells in group 1 ILC1s showed a significant decrease in the hepatic pre-metastatic niche of PDAC-bearing mice. Next, we tried to investigate the effects of pancreatic cancer-derived exosomes on NK cells. Firstly, we found that pancreatic cancer-derived exosomes could be incorporated by NK cells. Nevertheless, it was still uncertain whether pancreatic cancer-derived exosomes could mediate immune suppression upon co-incubation with NK cells.

The stress proteins MICA and MICB are commonly expressed by many human cancers due to genomic damage[118]. NKG2D is a key activating receptor for NK cell cytotoxicity[104]. The binding of MICA/MICB to NKG2D receptors triggers NK cell mediated-cytotoxicity and enables them to eliminate cancer cells[119]. Our result indicated that the expression of NKG2D on NK cells was significantly downregulated by pancreatic cancer-derived exosomes. This result was consistent with previous research[120]. After recognition and activation, NK cells synthesize and release effective cytokines into the tumor cells. For example, IFN-γ and TNF-α are two indispensable cytokines for NK cell cytotoxicity. Neutralization of IFN-γ and TNF-α

significantly impaired NK cell activity[106]. We demonstrated that exosomes treatment led to less production of IFN-γ and TNF-α in NK cells. In addition, as a functional marker for NK cell activity, the expression of CD107a was also significantly downregulated. Recently, the importance of cellular metabolism of immune cells has gained increasing attention[121]. Enough nutrients and energy are essential for NK effector functions[122]. Cong J *et al*. demonstrated that the tumor could reduce NK cell glycolytic capacity, which resulted in reduced cytotoxicity and NK cell dysfunction[67]. We found that NK cells exhibited less CD71 and CD98, as well as reduced glucose uptake ability after exosomes treatment. Dysregulated metabolism caused by pancreatic cancer-derived exosomes affected multiple biological processes in NK cells, such as interfered protein synthesis and impaired energy production. Our result was consistent with previous studies investigating the effects of tumor-derived exosomes on immune cells[115, 123, 124]. Our findings suggested that pancreatic cancer-derived exosomes induced a dysfunctional phenotype of NK cells, which ultimately contributed to an immunosuppressive microenvironment in the pre-metastatic niche.

Pancreatic cancer cells with high aldehyde dehydrogenase 1 (ALDH1) expression are considered as cancer stem cells (CSCs)[125]. Ames *et al*. demonstrated that ALDH1^{bright} cells had greater surface expression of ligands for the NK activation receptor, NKG2D and NK cells preferentially targeted CSCs[108]. Here, we found that after enrichment using sphere formation assay, pancreatic CSCs exhibited high expression of ligands for NKG2D. However, pancreatic cancer-derived exosomes impaired NK cell cytotoxicity against CSCs. CSCs are often thought to be responsible for tumor metastasis[126]. The inhibition of NK cell cytotoxicity allowed pancreatic CSCs to escape from NK cell immune surveillance and colonize in the target organ.

TGF-β signaling pathway is involved in the regulation of fibroblast activation, epithelial to mesenchymal transition (EMT), angiogenesis and immunosuppression in cancer[127-129]. As a key inhibitory cytokine, TGF-β1 plays a dominant role in modulating NK cell function[107, 130]. For example, TGF-β1 attenuated NK cell

responses by downregulating NKG2D expression in patients with advanced cancer[131]. Among various signals delivered by pancreatic cancer-derived exosomes to NK cells, TGF-β1 was thought to be a candidate responsible for NK cell dysfunction. Our results showed that TGF-β1 impaired NK cell function, including downregulated expression of NKG2D, CD107a, CD71 and CD98, decreased production of cytokines, such as TNF-α and IFN-γ, as well as reduced glucose uptake ability. Generally speaking, activation of TGF-β/Smad2/3 signaling pathway is implicated in NK cell dysfunction[109]. We found that either pancreatic cancer-derived exosomes or TGF-β1 could induce the phosphorylation of Smad2/3 in NK cells. However, the phosphorylation level of Smad2/3 returned to the baseline in the presence of SB-431542. Therefore, we proposed that pancreatic cancer-derived exosomes inhibited NK cell function via the TGFβ1-Smad2/3 pathway. Pancreatic cancer-derived exosomes delivered TGF-β1 to the surface of NK cells, binding to the TGFβ receptors (TGFβRI/II). Activation of TGFβRI/II by TGF-β1 induced the phosphorylation of serine/threonine residues and triggered phosphorylation of Smad2/3. Then phosphorylated-Smad2/3 translocated to the nucleus and regulated gene transcription, thereby modulating NK cell function[132]. As a TGFβRI inhibitor, SB-431542 exhibits cytotoxicity against pancreatic cancer cells *in vitro*[133]. Further investigations are needed to explore its anti-tumor effect *in vivo*, especially its influence on phenotypic and functional diversity of NK cells.

Without specific symptoms, it is a major challenge to detect PDAC at early stages. Exosomes are promising to be developed as a liquid biopsy tool for early detection and diagnosis in PDAC[134]. Recently, it's reported that the levels of exosomal PD-L1 in plasma, rather than soluble PD-L1, were associated with disease progression in patients with head and neck squamous cell carcinomas (HNSCCs)[135]. In our study, we measured the levels of serum exosomal TGF-β1 in PDAC patients. Interestingly, compared to healthy donors, serum exosomal TGF-β1 was significantly elevated in PDAC group. Therefore, serum exosomal TGF-β1 holds promise to be used as a

diagnostic tool for detection of PDAC.

Apart from TGF-β1, pancreatic cancer-derived exosomes also contained multiple other immune regulatory factors, such as PVR and Nectin-2, which could be delivered as inhibitory signals to NK cells. Both PVR and Nectin-2 can bind to inhibitory receptors on NK cell, including CD96, PVRIG and TIGIT[70]. Therefore, in addition to TGF-β1, PVR and Nectin-2 in pancreatic cancer-derived exosomes could also impaired NK cell function. It has been demonstrated that PVR/Nectin-2-TIGIT axis is involved in attenuated NK cell cytotoxicity[136]. As a checkpoint receptor, blockade of TIGIT prevented NK cell dysfunction and elicited NK cell anti-tumor responses in tumor-bearing mouse models[137]. Immune checkpoint inhibitors targeting CTLA-4, PD-1, and PD-L1 have shown clinical benefit for patients with non-small cell lung cancer (NSCLC), advanced melanoma and several other cancers[138-140]. However, these inhibitors are less effective in patients with PDAC[141]. Our result indicates that TIGIT offers a potential immunotherapeutic target in PDAC.

Nevertheless, a limitation of the present study was that *in vivo* effects of human pancreatic cancer-derived exosomes on NK cells was not investigated. To address this issue, humanized patient-derived xenograft mouse models, which can better recapitulate tumor heterogeneity and simulate complexity of immune system, serve as a better platform for further investigation[142].

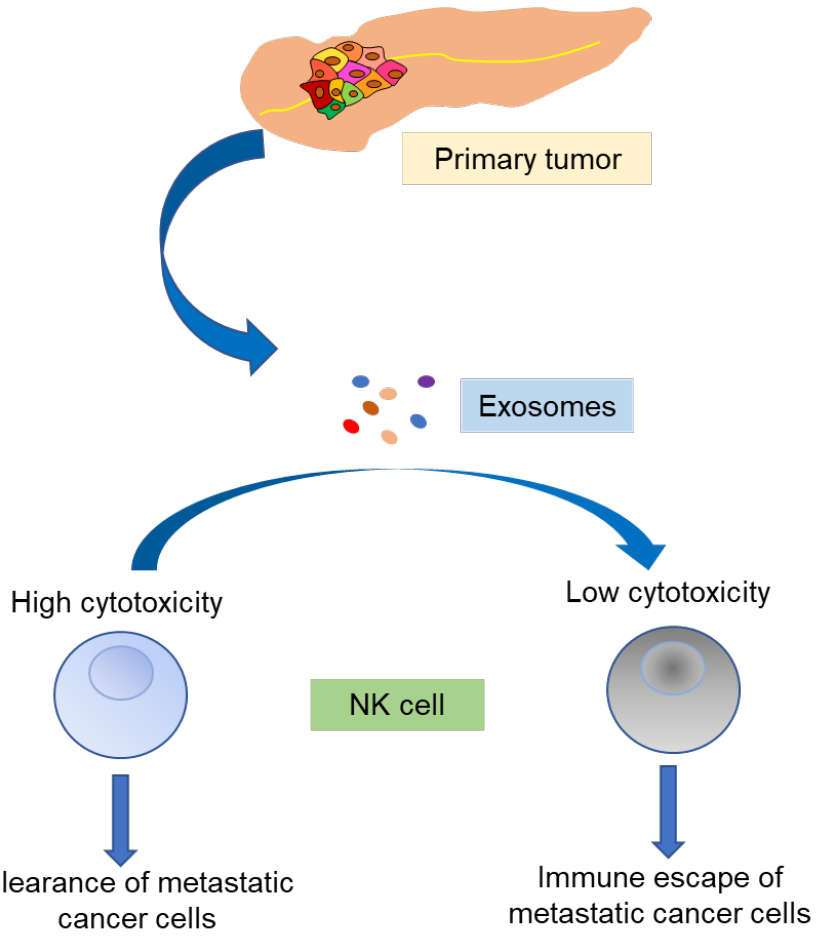

Figure 13. The role of tumor-derived exosomes in hepatic pre-metastatic niche formation of PDAC

In conclusion, we propose a novel mechanism of immune escape in PDAC. Pancreatic cancer can establish a pre-metastatic niche in the liver via tumor-derived exosomes. Pancreatic cancer-derived exosomes carrying immunosuppressive cargos mediate NK cell dysfunction. Metastatic pancreatic cancer cells evade immune surveillance of NK cells and ultimately generate metastases in the liver (Fig. 13). Additionally, serum exosomal TGF-β1 may represent a promising non-invasive diagnostic tool in PDAC.

VI. SUMMARY

Here, tumor-derived exosomes were isolated from a highly metastatic pancreatic cancer cell line L3.6pl and a PDAC patient derived primary cancer cell line TBO368 to investigate the proteomic profile of exosomes. Distant metastases of PDAC tend to occur predominantly in liver and lung. We found that adhesion receptors, especially integrins like ITGAV and ITGβ5, which are associated with organ-specific metastases in PDAC, were enriched in pancreatic cancer-derived exosomes. In addition, these exosomes displayed a variety of immune regulatory factors, such as TGF-β1, Nectin-2 and PVR. Therefore, we co-cultured NK cells with exosomes derived from pancreatic cancer cells. The expression of NKG2D, CD107a, TNF-α and INF-γ in NK cells was significantly downregulated. NK cells also exhibited reduced expression of CD71 and CD98, as well as impaired glucose uptake ability. Additionally, NK cell cytotoxicity against pancreatic CSCs was attenuated. Moreover, pancreatic cancer-derived exosomes induced the phosphorylation of Smad2/3 in NK cells. Compared to healthy donors, serum exosomal TGF-β1 was significantly increased in patients with PDAC. In conclusion, our findings suggest that tumor-derived exosomes mediate NK cell dysfunction in the pre-metastatic niche of PDAC.

VII. ZUSAMMENFASSUNG

In dieser Arbeit wird gezeigt, dass die Dysfunktion von NK-Zellen (Natural Killer) in der prä-metastatischen Nische der Leber beim duktalen Adenokarzinom des Pankreas durch tumor-spezifische Exosomen vermittelt wird. Dazu wurden aus einer hochmetastatischen Pankreaskarzinom-Zelllinie, L3.6pl, und einer Primärzelllinie aus dem Pankreaskarzinom eines Patienten, TBO368, Exosomen isoliert. Mit Hilfe einer Proteomanalyse konnte gezeigt werden, dass Adhäsionsrezeptoren, insbesondere Integrine wie ITGAV und ITGβ5, die mit organspezifischen Metastasen im Pankreaskarzinom assoziiert sind, in den Exosomen angereichert waren. Darüber hinaus wiesen diese Exosomen eine Vielzahl von immunregulatorischen Faktoren wie TGF-β1, Nectin-2 und PVR auf. Darauffolgend wurden NK-Zellen mit Exosomen, welche aus dem Zellkulturüberstand der Zelllinie L3.6pl isoliert wurden, kultiviert. Die Expression von NKG2D, CD107a und INF-γ in den NK-Zellen war signifikant herunterreguliert. Die NK-Zellen zeigten ebenfalls eine verminderte Expression von CD71 und CD98. Zusätzlich führten die Kultivierung mit tumor-spezifischen Exosomen zur Phosphorylierung von Smad2/3 in den NK-Zellen. Im Vergleich zu gesunden Spendern war das exosomale TGF-β1 im Serum bei Patienten mit Pankreaskarzinom signifikant erhöht. Zusammenfassend beschreiben unsere Ergebnisse die immunsuppressiven Effekte von tumor-spezifischen Exosomen und liefern neue Erkenntnisse zu unserem Verständnis von NK-Zelldysfunktion in der prä-metastatischen Nische des duktalen Adenokarzinoms des Pankreas.

VIII. ABBREVIATION

2-NBDG, 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose

ADCC, antibody-dependent cellular cytotoxicity

ALDH1, aldehyde dehydrogenase 1

bFGF, basic fibroblast growth factor

CAFs, cancer associated fibroblasts

CSCs, cancer stem cells

CTCs, circulating tumor cells

DAPI, 4', 6-Diamidin-2-phenyl-Indol

DMEM, Dulbecco's modified eagle medium#

DMEM, Dulbecco's Modified Eagle's Medium

DMSO, dimethyl sulfoxide

DNA, desoxyribonucleic acid

DPBS, Dulbecco's phosphate-buffered saline

DTCs, disseminated tumor cells

ECM, extracellular matrix

EGF, epidermal growth factor

ELISA, enzyme-linked immunosorbent assay

EMT, epithelial-mesenchymal transition

EVs, extracellular vesicles

FACS, fluorescence activated cell scan

FBS, fetal bovine serum

FC, flow cytometry

FCS, fetal calf serum

GAPDH, Glyceraldehyde 3-phosphate dehydrogenase

GO, Gene Ontology

HGF, hepatocyte growth factor

HSCs, hepatic stellate cells

HUVECs, human umbilical vein endothelial cells

IF, immunofluorescence

IF, immunofluorescence

IFN-γ, interferon-gamma

IHC, immunohistochemistry

IL-10, interleukin-10

IL-2, interleukin-2

IL-6, interleukin-6 (IL-6),

KCs, Kupffer cells

MDSCs, myeloid-derived suppressor cells

MEM, Minimum Essential Media

MFI, mean fluorescence intensity

MICA, MHC class I chain-related protein A

MICB, MHC class I chain-related protein B

MMP, matrix metalloproteinase

MS, mass spectrometry

MS, mass spectrometry

NEAA, Non-Essential Amino Acids

NK cells, natural killer cells

NKG2D, natural killer group 2D

NSG, NOD scid gamma mouse

NTA, nanoparticle tracking analysis

NTA, nanoparticle tracking analysis

PDAC, pancreatic ductal adenocarcinoma

PGE2, prostaglandin E2

PSCs, pancreatic stellate cells

PVR, poliovirus receptor

RPMI, Roswell Park Memorial Institute

SEER, the Surveillance, Epidemiology, and End Results

STAT3, signal transducer and activator of transcription 3

TBST, Tris Buffered Saline with Tween 20

TEM, transmission electron microscope

TGFbRI, TGF beta receptor 1

TGFbRII, TGF beta receptor 2

TGF-β1, transforming growth factor beta 1

TIGIT, T-cell immunoglobulin and ITIM domain

TME, tumor microenvironement

TNF-α, tumor necrosis factor-alpha

Tregs, regulatory T cells

VEGF, vascular endothelial growth factor

WB, Western blot

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