Aus der

Klinik für Allgemein-, Viszeral-, und Transplantationschirurgie

Klinikum der Ludwig-Maximilians-Universität München

CD96 as a potential immunotherapy target for pancreatic cancer

Dissertation
zum Erwerb des Doktorgrades der Medizin
an der Medizinischen Fakultät
der Ludwig-Maximilians-Universität München

vorgelegt von
Shikai Feng

aus
Chongqing

Jahr
2025

Mit Genehmigung der Medizinischen Fakultät der
Ludwig-Maximilians-Universität München

Erstes Gutachten:	Prof. Dr. Alexandr Bazhin
Zweites Gutachten:	Priv. Doz. Dr. Ivonne Regel
Drittes Gutachten:	Priv. Doz. Dr. Michael Hristov

Dekan: Prof. Dr. med. Thomas Gudermann

Tag der mündlichen Prüfung: 13.10.2025

Table of content

Zusammenfassung (Deutsch):

Bauchspeicheldrüsenkrebs ist ein hoch invasiver bösartiger Tumor, der zunächst kaum Symptome zeigt und bei später Diagnose eine sehr geringe Überlebensrate aufweist. Trotz Fortschritten bei den bestehenden Behandlungsmethoden ist die Langzeitüberlebensrate weiterhin niedrig, was den dringenden Bedarf an neuen Therapieansätzen unterstreicht. Die Immuntherapie als Behandlungsstrategie gegen die Tumorevasion hat in den letzten Jahren bei verschiedenen malignen Tumoren deutliche Fortschritte gemacht. Das Ansprechen des Pankreaskarzinoms auf die Immuntherapie ist jedoch begrenzt, weshalb die Suche nach weiteren potenziellen Therapiezielen von großer Bedeutung ist.

CD96 ist ein transmembranes Immunglobulin, dessen wichtigster Ligand CD155 ist und das hauptsächlich auf NK-Zellen und $CD8^+$ T-Zellen exprimiert wird, und reguliert die Immunantwort. In jüngster Zeit wurde jedoch festgestellt, dass CD96 in verschiedenen Tumoren, einschließlich Bauchspeicheldrüsenkrebs, überexprimiert wird. CD96 spielt eine wichtige Rolle bei Prozessen wie Tumorevasion und Metastasierung. Ziel dieser Studie ist es, das Potential von CD96 als immuntherapeutisches Ziel bei Bauchspeicheldrüsenkrebs zu untersuchen, indem die Expression von CD96 in Immunzellen, die Expression von CD155 in Bauchspeicheldrüsenkrebszellen und die Expression von IFN-γ nach Zugabe von rekombinantem CD155-Protein und CD96-Antikörpern in PBMCs analysiert wird.

Die Forschungsergebnisse zeigen, dass CD155 in großen Mengen auf der Oberfläche von Pankreaskrebszellen exprimiert wird, während CD96 in großen Mengen auf der Oberfläche von $CD8^+$ T-Zellen exprimiert wird. Die Zugabe von rekombinantem CD155-Protein zu PBMCs, die T-Zellen enthalten, führt zu einer Abnahme der Effektorfähigkeit der $CD8^+$ T-Zellen, was jedoch durch die Hemmung der Bindung zwischen CD96 und seinem Liganden CD155 teilweise wiederhergestellt werden kann.

Basierend auf diesen Ergebnissen wurde CD96 als ein potenzieller Angriffspunkt für eine Immuntherapie bei Bauchspeicheldrüsenkrebs identifiziert. Die Blockade von CD96 könnte die Immunabwehrstrategie des Tumors umkehren und die Effektorfähigkeit des körpereigenen Immunsystems wiederherstellen, um das Wachstum und die Metastasierung des Tumors zu unterdrücken. Um die Übertragung von CD96 als klinisches Therapieziel zu erreichen, ist es jedoch notwendig, die molekularen Mechanismen von CD96 im Immunabwehrsystem des Bauchspeicheldrüsenkrebses im Detail zu verstehen und konkrete Behandlungsmethoden zu erforschen.

Zusammenfassend wurde CD96 als potenzielles immuntherapeutisches Ziel bei Bauchspeicheldrüsenkrebs identifiziert. Immuntherapeutische Strategien, die auf CD96 abzielen, haben das Potenzial, die Prognose und das Überleben von Patienten mit Pankreaskarzinom zu verbessern. Die klinische Anwendung erfordert jedoch weitere Forschung und Validierung, unter anderem präklinischer und klinischer Studien. Ein besseres Verständnis der immunregulatorischen Mechanismen von CD96 könnte neue Wege für die Immuntherapie des Pankreaskarzinoms eröffnen.

Abstract (English):

Pancreatic cancer, as a highly invasive malignant tumor, exhibits inconspicuous early symptoms, resulting in extremely low survival rates on late-stage diagnosis. Despite some progress in existing treatment methods, long-term survival rates remain low, highlighting the urgent need for new therapeutic strategies. Immunotherapy, a treatment strategy targeting tumor immune evasion mechanisms, has made significant breakthroughs in various malignant tumors in recent years. However, pancreatic cancer shows a limited response to immunotherapy, underscoring the crucial significance of identifying additional potential treatment targets.

CD96, a transmembrane immunoglobulin with its primary ligand CD155, mainly found in NK cells and $CD8^+$ T cells, participating in immune response regulation. Recent research has found high expression of CD96 in various tumors, including pancreatic cancer. CD96 is vital for processes like tumor immune evasion, invasion, and metastasis. This study examines CD96's expression in immune cells, CD155 expression in pancreatic cancer cells, and IFN-γ expression following the addition of CD155 recombinant protein and CD96 antibodies to PBMCs, with the goal of exploring CD96's possibility as an immunotherapy point for pancreatic cancer.

The results indicate that pancreatic cancer cells abundantly express CD155 on their surface, while $CD8^+$ T cells exhibit high surface expression of CD96. Adding CD155 recombinant protein to PBMCs reduces the IFN-γ expression by $CD8^+$ T cells, which could be partially restored by blocking the connection between CD96 and its ligand CD155.

Based on these findings, we identify CD96 as a potential target point for immunotherapy in pancreatic cancer. Blocking it may reverse the tumor's immune evasion strategy, restoring the capacity of the patient's immune system to inhibit tumor growth. However, the transformation of CD96 into a clinical treatment target requires a deeper understanding of its molecular mechanisms in the pancreatic cancer immune evasion system, along with the

exploration of specific treatment methods.

In conclusion, CD96 could be recognized as a potential immunotherapy target for pancreatic cancer. Through immunotherapeutic strategies targeting CD96, there is optimism for improving the prognosis and survival rates of individuals with pancreatic cancer. However, further research and validation, including preclinical studies and clinical trials, are still necessary for clinical application. A comprehensive grasp of the immune regulatory mechanisms of CD96 and the optimization of treatment strategies may pave the way for new avenues in the immunotherapy of pancreatic cancer.

List of figures

List of tables

List of abbreviations

5-FU	5-fluorouracil
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
cDNA	Complementary DNA
dFdCP	Difluorodeoxycytidine diphosphate
DMEM	Dulbecco's modified eagle medium
DPBS	Dulbecco's phosphate-buffered saline
EDTA	Ethylenediaminetetraacetic acid
Extra.	Extracellular
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FMO	Fluorescence minus one
GAPDH	Glycerinaldehyd-3-phosphat-Dehydrogenase
GEPIA	Gene expression orofiling interactive analysis
gDNA	Genomic DNA
ICB	Immune checkpoint blockade
ICC	Immunocytochemistry
IOD	Integrated optical density
IFN-γ	Interferon gamma、interferon-γ
IgSF	Immunoglobulin superfamily
Intra.	Intracellular
ITIM	Immunoreceptor tyrosine-based inhibitory motif
MFI	median fluorescence intensity
OCR	Oxygen consumption rate
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PTPs	Protein tyrosine phosphatases
qPCR	Quantitative polymerase chain reaction
ROS	Reactive oxygen species
SD	Standard deviation

Stim.	Stimulated
TBS	Tris buffered saline
TBST	TrisBufferedSaline with Tween-20
TCR	T cell receptor
TME	Tumor microenvironment
Unstim.	Unstimulated

1. Introduction

1.1 Present status of pancreatic cancer

Pancreatic cancer has been recognized to be one of the fatal cancer phenotypes. Pancreatic cancer is a type of malignancy that arises from the malignant transformation of pancreatic cells. These tumor cells can grow rapidly, occupying the living space of normal cells, and invade other tissues [1-2]. The most common type is exocrine adenocarcinoma, accounting for around 85% of cases, followed by endocrine carcinoma (5%) [3]. Despite poor understanding of its specific etiology, it has been revealed to be induced by factors including smoking history, age, and obesity. Patients with advanced pancreatic cancer may experience obvious symptoms such as weight loss, stomach discomfort, digestive issues, itching of the skin and jaundice [4-5]. However, pancreatic cancer often presents with no obvious symptoms or signs at the early stage. Moreover, its symptoms may resemble other diseases such as gastritis or peptic ulcers, leading to misdiagnosis frequently. Once diagnosed, pancreatic cancer cells may have already spread to or invade other tissues and organs, resulting in poor prognostic outcomes [6-7].

Although pancreatic cancer is not among the most common cancers, it ranks seventh globally in terms of mortality (Figure 1). The morbidity and mortality of pancreatic cancer are the highest in North America and Europe (Figure 2). In 2018, the number of newly diagnosed pancreatic cancer reached approximately 460,000 casesd worldwide, with 432,242 reported deaths, indicating a remarkably high mortality [8-9]. Pancreatic cancer patients may experience significantly improved postoperative outcomes with advancements in medical techniques [10]. According to a statistical estimation in the Europe and the US in the 1990s, about 95% of patients who developed pancreatic cancer died within five years following surgery, but this mortality decreased to 91% by 2019 [11-12]. The proposed high mortality in pancreatic cancer patients is mainly attributed to its diagnosis at

advanced stages. Therefore, it highlights the necessity and urgency of late-stage patient treatment to improve their survival [13].

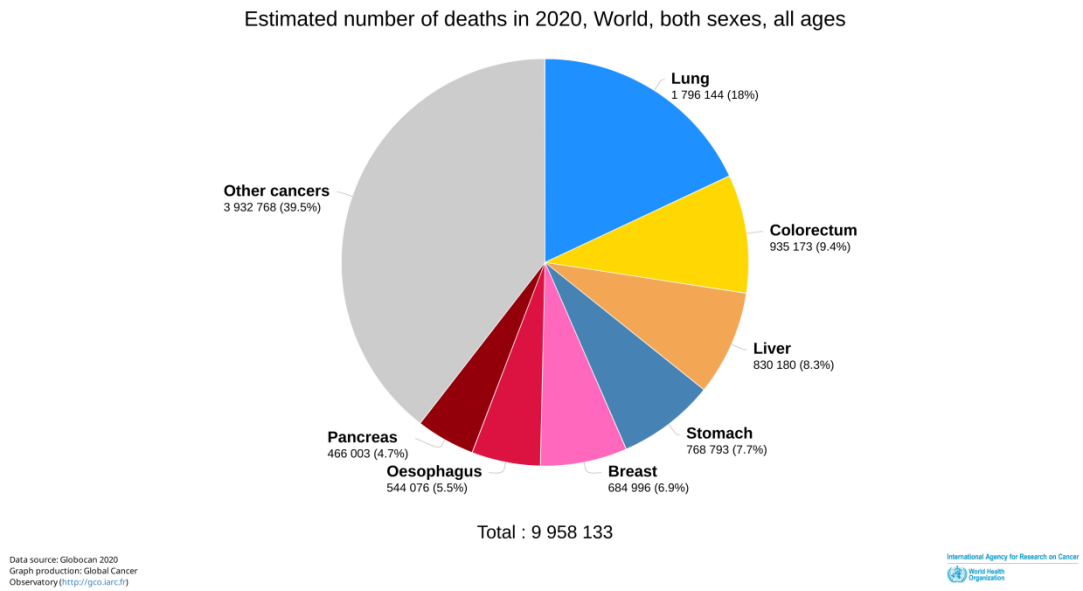

Figure 1: Estimated number of deaths of different cancers around the world in 2020

Data sources: GLOBOCAN 2020 (https://gco.iarc.fr/)

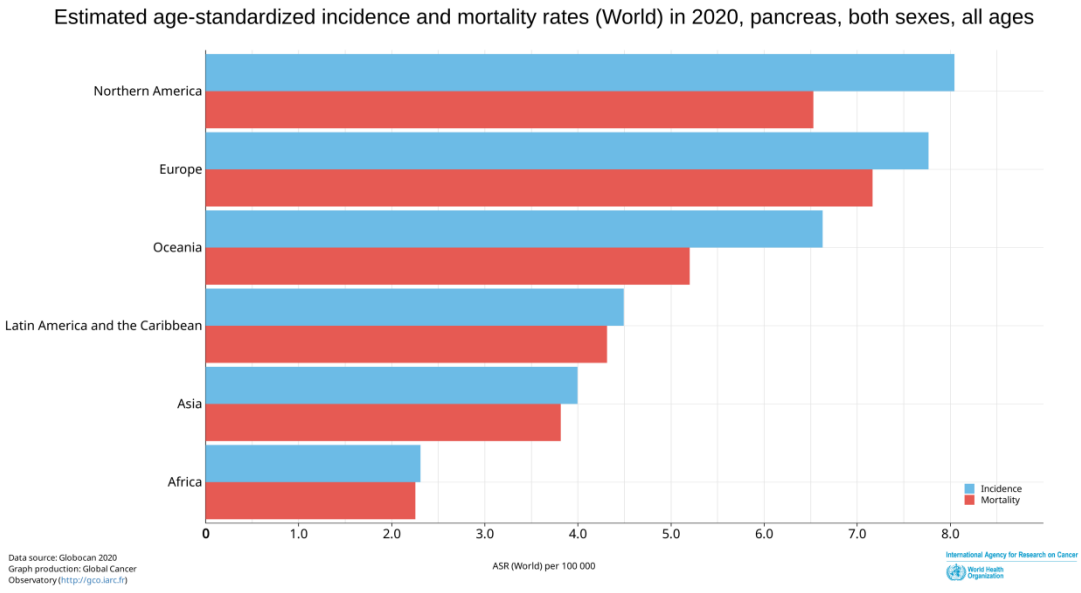

Figure 2: Estimated age-standardized incidence and mortality rates of pancreatic cancer globally in 2022

Data sources: GLOBOCAN 2020 (https://gco.iarc.fr/)

1.2 Treatment for pancreatic cancer

Cancer treatment has always been a topic of concern for medical experts. Its prevention and control can be broadly categorized into three stages: primary prevention; the detection and treatment in early-stage malignancy; and improvement of life quality along with extending survival time [14]. Generally, the second stage is usually a focus for healthcare institutions. Anatomically, pancreatic cancer is featured by deep alocation in the abdomen, no typical early symptoms, and high risk of hematogenous metastasi owing to vascular invasion, leading to higher possibility of diagnosis at the advanced stages [15]. Hence, to realize effective pancreatic cancer treatment, the key lies in early screening and diagnosis. In actual clinical practice, the concept of early detection, despite appealing, reveal inferior clinical application. An American preventive medicine institution has given a pancreatic cancer screening program a rating of just "D" in the general population, due to its extremely low incidence (approximately 1/10,000), the associated high costs, and psychological burden of false positives [16]. Thus, a more feasible approach involves screening individuals whose family members have medical history of pancreatic cancer, pancreas cystic lesion, diabetes, persistent pancreatitis, and over 50-year-old populations. Methods available for early diagnosis of pancreatic cancer include medical imaging, pathological examination, serological testing, and liquid biopsy [17-18].

It is critical for screening high-risk populations of pancreatic cancer. However, efficient, and appropriate treatments can also contribute to lowering the death rate of individuals diagnosed with advanced pancreatic cancer, while also improving their quality of life after diagnosis [19].

Surgery is currently the only therapeutic option with the possibility of cure for patients with pancreatic cancer diagnosed at an advanced stage [13,20]. In 1882, Professor Friedrich Trendelenberg in Germany performed the world's first pancreatic cancer resection [21]. Although the patient died from postoperative complications, subsequent global improvements and refinements in surgical techniques have significantly enhanced the

postoperative survival of patients. However, the overall post-operative 5-year survival rate is still below 20%, necessitating complementary treatment strategies [22-23].

When surgery is feasible for some patients, a common treatment approach involves surgery followed by postoperative chemotherapy [24]. Currently, chemotherapy for pancreatic cancer patients has made great progress, although there is still a lack of specific targeted chemotherapy drugs for pancreatic cancer [25]. Generally, chemical agents are used during chemotherapy to kill actively proliferating tumor cells. However, its non-selective toxicity may disturb the function of normal cells, triggering side effects such as myelosuppression, gastrointestinal reactions, liver damage, neurotoxicity, and renal toxicity [26]. As an FDA-authorized drug for pancreatic cancer treatment, gemcitabine can act by inhibiting DNA synthesis through the production of its metabolite difluorodeoxycytidine diphosphate [27]. In a clinical trial (1997) on the efficacy of gemcitabine, nearly a quarter of pancreatic cancer patients had a better rate of post-treatment survival compared the control group [28]. The relatively inferior clinical outcome may be explained by the low tumor vascularization and poor tumor stromal permeability, which may block the penetration of drugs to the central region of tumor [29]. It was reported that gemcitabine combined with other drugs, such as albumin-bound paclitaxel, would improve the clinical outcomes [30]. Another important therapeutic agent for pancreatic cancer is 5-fluorouracil (5-FU), which primarily limits the production of thymidine, an essential component in reproduction of DNA. Despite nonobvious effect when used alone, 5-FU combined with other chemotherapeutic agents has achieved promising outcomes [31]. In 2011, Conroy et al. evaluated a combined therapy known as "FOLFIRINOX", showing significantly improved postoperative survival time for pancreatic cancer patients compared to gemcitabine-only treatment group. However, the "FOLFIRINOX" resulted in more adverse reactions, neutropenia especially. Overall, the "FOLFIRINOX" may be a more effective and preferable

therapeutic option for advanced pancreatic cancer. However, its higher number of combined drugs may result in more significant side effects, mainly gastrointestinal reactions such as malignant vomiting and diarrhea [32]. In addition, other regimens may also induce varying degrees of side effects, such asneutropenia or thrombocytopenia caused using gemcitabine alone [30]. Altogether, rational use of chemotherapeutic agents can extend the survival of patients to some extent yet accompanied by possible side effects. It may lead to the presence of additional ailments in these patients, preventing them from achieving a normal quality of life. Therefore, it is crucial to develop more effective drugs with fewer side effects.

There have been rapid advancements in immunotherapy recently, including progress in immunotherapeutic approaches for pancreatic cancer, which will be introduced in detail as follows:

1.3 The application of immunotherapy in pancreatic cancer

Immunotherapy in pancreatic cancer can be roughly classified as cancer vaccines, CAR-T cell therapy, and immune checkpoint blockade (ICB) [33].

1.3.1 Cancer vaccines

Cancer vaccines can carry tumor-specific antigen to immune cells using tumor cells, peptide, dendritic cells, DNA, or microRNAs. Immune cells can be activated, upon receiving antigen information, and subsequently eliminate relevant tumor cells [34]. Earlier studies have documented preliminary therapeutic effects in patients using pancreatic cancer vaccines [35-37]. However, no such therapeutic effects on pancreatic cancer patients were observed in later clinical trials. In a Phase III trial, adjuvant vaccine named “algenpantucel-L” showed no significant postoperative benefits for pancreatic cancer patients [38]. In another Phase III trial, a GV1001 vaccine derived from tumor-associated autoantigen human telomerase reverse transcriptase produced no favorable clinical outcomes when compared with chemotherapy [39]. In addition, a type of allogeneic pancreatic cancer vaccine (GVAX) did not show significant therapeutic effect when used

together with chemotherapy versus chemotherapy alone [40]. Overall, the clinical efficacy of cancer vaccines remains modest when applied for treating pancreatic cancer.

1.3.2 CAR-T cell therapy

CAR-T cell therapy is another cancer immunotherapy that involves genetic modification of patient-derived T cells to express chimeric antigen receptors which can identify antigens on cancer cells. This activation enables T cells to kill tumor cells, with confirmed effect on blood cancers, but no similar function in solid malignancies including pancreatic cancer [41-42]. Challenges for CAR-T cell therapy in solid tumors include physical barriers created by abundant fibrotic tissue in the tumor microenvironment (TME), shared antigens between tumors and normal tissues, immunosuppressive TME, and late-stage diagnosis of pancreatic cancer with widespread metastasis [43]. Thus, there is still moderate outlook for CAR-T cell therapy currently.

1.3.3 ICB therapy

T cells perform a vital part among anticancer immune system reactions [44]. They express co-stimulatory or co-inhibitory molecules, also known as immune-mediated checkpoints, which can regulate the proliferation and differentiation of T cells [45]. In the process of tumor growth, most of immunological checkpoints can decrease T cell responses while preventing immune surveillance [46]. The immunological checkpoints PD-1 and CTLA-4 are well-studied immune checkpoints in the last years [47-48]. Supported by encouraging results about ICB therapy in solid tumors, drugs targeting PD-1 (Nivolumab and Pembrolizumab) and those targeting CTLA-4 (Tremelimumab and Ipilimumab) have been approved by FDA for melanoma treatment [49]. Nivolumab alone can effectively increase the prognosis of patients with skin cancer, lung carcinoma, or renal carcinoma [50]. However, ICB monotherapy failed to show effective clinical outcomes in individuals with pancreatic cancer [51]. But more effective results were reported when combined with different checkpoint blockers. For example, in a Phase II trial,

Tremelimumab combined with Durvalumab outperformed Durvalumab monotherapy in the aspect of clinical response for patients with pancreatic cancer [52]. Therefore, combination ICB-based combined therapy is more effective than monotherapy, yet with limited well-studied checkpoints (e.g., PD-1 and CTLA-4) currently. The development of more immune-mediated checkpoint blockers, such as CD96, holds significant promise for providing additional therapeutic options for pancreatic cancer patients.

1.4 CD96

1.4.1 Structure of CD96

The immunoglobulin superfamily (IgSF), a concept emerged in the previous century, is a protein family with structural and functional relevance, functioning significantly in the immune system and other intercellular interactions. Members of the IgSF have one or more extracellular immunoglobulin domains [53]. CD96 from the IgSF, commonly referred to as TACTILE, was identified by PL Wang et al. in 1992 [54]. In SDS-PAGE assays, CD96 appeared as a 160 kDa protein under reducing condition and as 160, 180, or 240 kDa under non-reducing conditions. The nucleotide sequence encoding CD96 is 1.5 kb in length, starting with the ATG initiation codon [54]. CD96 is composed of an external area, a neck area, a domain of the transmembrane, and a cytosolic area [55]. In general, the extracellular domain of immunoglobulins can be categorized into three sub-domains of C, V, and H domains, with 55-60, 65-75, and 35-55 residues between cysteine residues, respectively [54]. CD96 has three domains, with two subtypes possessing different Ig folding in the second domain by alternative splicing in humans [56]. Subtype 1 has 568 amino acids in the extracellular region, with V-V-C domains, while subtype 2 owns either an I or C domain in the second Ig domain, resulting in V-I/C-C domains [56]. Mouse CD96 shares similar extracellular structure with the second subtype of human CD96 [55]. CD96 has a flexible neck area close to the transmembrane region which are abundant with serine, proline and threonine residues and a rod-shaped structure [57]. The transmembrane domain of CD96 is a shared domain among other members of the IgSF. The cytosolic region contains a YXXM motif (note: not existed in mouse CD96), an ITIM motif, and a P-rich area

[55] (Figure 3).

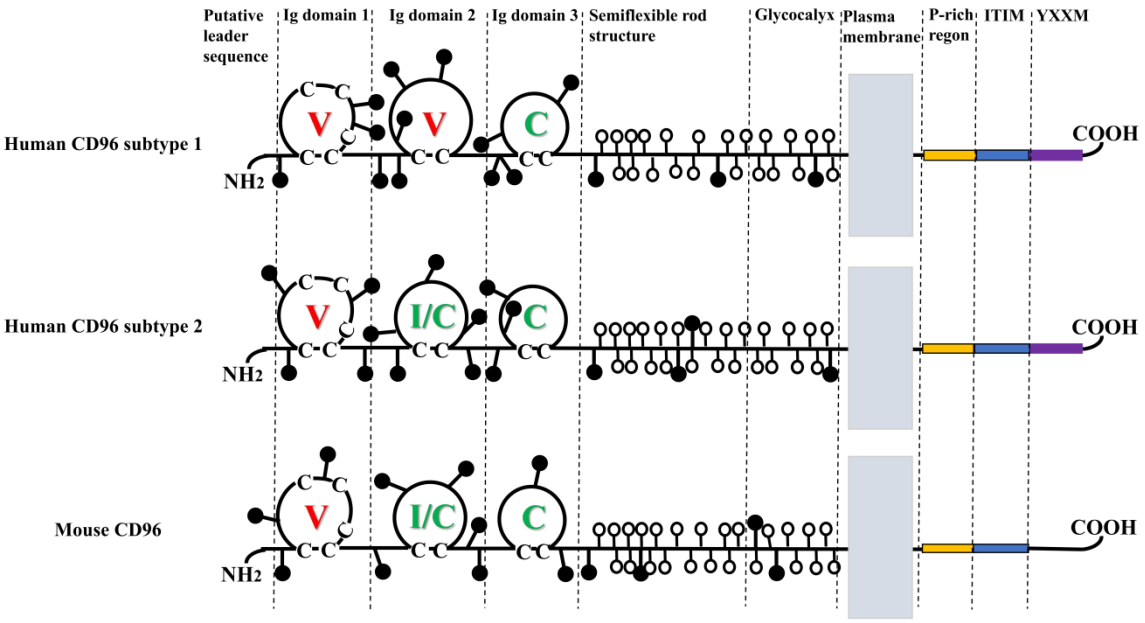

Figure 3: Structure of CD96

The picture comes from one of our reviews piblished previously: Feng S, Isayev O, et al. CD96 as a Potential Immune Regulator in Cancers. *Int J Mol Sci*. 2023 Jan 9;24(2):1303.

1.4.1.1 The ITIM and YXXM motifs in the CD96 structure

The C domain of human CD96 structure has two different motifs of ITIM and YXXM, while mouse CD96 contains ITIM motif only [58]. Typically, the term motif is a nucleotide sequence pattern with specific biological functions, which is widely present in the genome and crucial in regulating gene expression or other biological processes. In gene regulation, proteins bind to specific nucleotide sequences to exert their specific functions. These specific nucleotide sequences, known as motifs, can exist in DNA or RNA. According to the category of negative inhibitory or positive stimulatory motifs [59-60], ITIM is a negative inhibitory motif, typically composed of 6-8 amino acids, including a pair of tyrosine residues that can inhibit signal transduction [61]. Immune receptors [e.g., antibody receptors or T cell receptors (TCR)] may be activated after binding to their respective ligands,

during which there may be phosphorylation of tyrosine residues of immune receptor. Phosphorylated ITIM can function as a signaling molecule to recruit protein tyrosine phosphatases (PTPs), enzymes eliminating phosphate groups on protein tyrosine residues to deactivate relevant signaling molecules. Therefore, ITIM acts as inhibitor in the immune system, which can downregulate cell activation status, keep immune balance, and prevent excessive activation that may trigger abnormal immune responses. This mechanism is important to prevent autoimmune diseases and maintain immune homeostasis [62]. Additionally, CD96 also contains a positive stimulatory motif of YXXM. These motifs are frequently observed in the cytosolic regions on TCR, which is an extracellular receptor recognizing antigens presented by major histocompatibility complexes. The tyrosine residues in the YXXM motif will be phosphorylated when TCR bind to an antigen and become activated. There will be an emergence of signaling transduction complex when phosphorylated YXXM binds to adapter proteins. Then, the formed complex can activate downstream signaling molecules to produce protein kinases and phosphatases, leading to the upregulation of intracellular signaling pathways, eventually the stimulation, expansion, and functional expression of T lymphocytes [63]. Generally, immune checkpoints have single effective motifs. For example, the PD-1 immune checkpoint has inhibitory motifs ITIM and ITSM; CTLA-4 has the inhibitory motif ITIM; and TIGIT has inhibitory motifs ITIM and ITT [64-66]. However, CD96 contains both negative inhibitory motif ITIM and the positive stimulatory motif TXXM. So far, there is still a poor understanding of the exact impact of CD96 on immune cell function, whether is inhibitory or stimulatory. Nevertheless, CD96 was reported to predominantly play an inhibitory role in immune cell function when binding with antigens [67-68]. For instance, CD96, in collaboration with PD-1, could downregulate the activity of $CD8^+$ T cells, and hence a combined use of CD96 and PD-1 blockade could be adopted for treating cervical cancer [69]. In addition, restricting the connection between CD96 along with its ligand CD155 can restore the immunotoxicity of NK cells against liver cancer [70]. At this stage,

further investigation is required to uncover the underlying reasons for these observations.

1.4.2 Expression of CD96

Initially, CD96 was identified due to its increased expression in the late stages of T cell activation [54]. CD96 appears primarily by $CD8^+$ T cells and $CD56^{bright}$ NK cells in human beings and mice. In $CD4^+$ T cells, CD96 was expressed in effector memory cells [71]. At mRNA level, CD96 was highly expressed NK and T cells, and moderately expressed in B cells, lymph nodes, etc, with little expression within the small intestine, granulocytes, bone marrow, and other tissues. CD96 protein production was detected primarily within cell membraneand in cytoplasm [72]. CD96 expression exhibited significant difference between healthy and tumor tissues based on data sourced from different databases. Specifically, different findings were observed in Oncomine and TCGA databases, both strong tools for obtaining cancer gene information. For example, Oncomine-sourced data indicated high CD96 mRNA levels in breast, brain, kidney, and blood cancers; while TCGA-sourced data revealed elevated CD96 mRNA levels in breast, leukemia, cervical, ovarian, endocervical, cholangiocarcinoma, colon, glioblastoma, renal, head and neck, liver, esophageal, pancreatic, melanoma, gastric, and testicular cancer. In addition, reduced CD96 mRNA expression was observed in lung cancer and thyroid cancer [72]. Furthermore, to clarify the potential role of CD96 expression in pan-cancer immune infiltration, the ESTIMATE method was employed to calculate the percentages of stromal cell populations, immune cells, and their combined percentages within the TME, represented by stromalscore, immunescore and estimatescore. Consequently, the study revealed strong correlations of CD96 expression with stromalscore in colon cancer, glioblastoma, and head and neck cancer; with immunescore in breast cancer, cervical cancer, endocervical cancer, and cholangiocarcinoma; as well as with estimatescore in cervical, colon, and esophageal cancer. All these discoveries supported the involvement of CD96 expression in the immune infiltration processes of malignant malignancies

[72]. In addition, according to the data retrieved from the TIMER 2.0, a database used to analyze immunological infiltration, CD96 expression was positively correlated with most immune cells, such as NK cells, neutrophils, and T cells; but negatively associated with myeloid-derived suppressive lymphocytes only. In other words, CD96 expression may play an essential part in the immunological invasion of most immune cells [72].

1.4.3 Ligand of CD96

CD155 is the major receptor of CD96. TIGIT, CD96 and CD226 have been documented to attach with the same receptor of CD155 to exhibit immune-regulatory roles [55] (Figure 4).

Comparable to the IgSF, CD155 has conservative amino acid and structural domain properties, which was first recognized to be a poliovirus receptor [73-74]. CD155 is the fifth member in the nectin-like molecule family, therefore also referred to as necl-5, relates to the nectin-like cellular relatives, comprising four nectins and five nectin-like molecular. These molecules, with features comparable to adhesion proteins, also also accepted as important players of cell attachment and division [75-77]. Furthermore, CD96 contains three extracellular domains, among which just the outer V region can attach to CD155, while the remaining second and third regions influence how they interact [56]. Although CD155 is rarely detected in normal tissues, it was measured with increased levels in melanoma, lung cancer, pancreatic cancer, etc. [78-82]. Meanwhile, the upregulation of CD155 in tumor tissues would usually predict poor prognosis [79-81].

Simultaneously, TIGIT acts as an inhibitory immunoglobulin protein found on NK and T cells, composing of an external immunoglobulin variable region, a transmembrane region, and a cytosolic region [83-85].

CD226 (or DNAM-1/Nectin-2), is a costimulatory checkpoint that can be detected in NK cells, T cells, monocytes/macrophages, dendritic cells, B cells, and various other cell types [86-89].

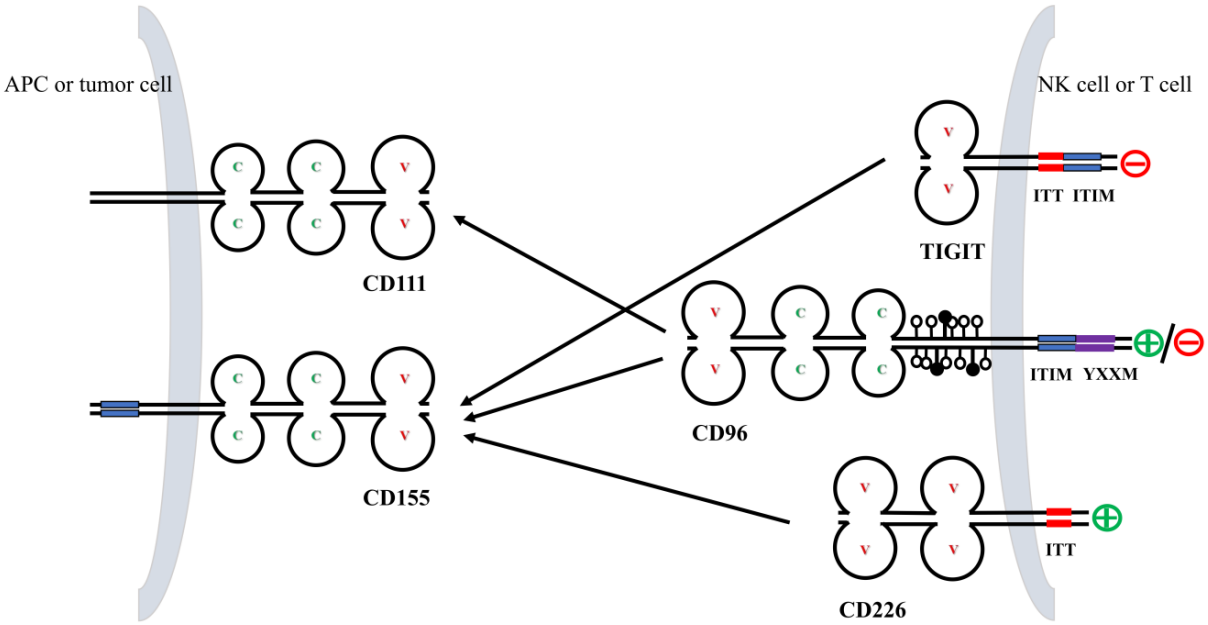

Figure 4: CD96 and it's ligand CD155

The picture comes from one of reviews published previously: Feng S, Isayev O, et al. CD96 as a Potential Immune Regulator in Cancers. *Int J Mol Sci*. 2023 Jan 9;24(2):1303.

1.4.4 Mechanisms of CD96 in regulating tumor immunity

1.4.4.1 CD96 regulates NK Cells

NK cells are innate lymphocytes, accounting for almost 15% of all circulatory lymphocytes, which can protect the immune system from the attack of viruses and cancer cells [91]. In general, human NK cells fall into two categories of $CD56^{bright}CD16^{-}$ (less developed and less effective cytokine generator) and $CD56^{dim}CD16^{+}$ (advanced type with cytotoxicity) [91-92]. Most of NK cells in bloodstreams are $CD56^{dim}CD16^{+}$, with $CD56^{bright}CD16^{-}$ accounting for <15% [91]. As for the types of molecules with different functions on the surfaces of NK cells, the stimulating molecules particularly connect to virus-infected or malignantly altered cells; while the inhibitory molecules can detect receptors on healthy cells and deliver inhibiting signals to NK cells, thus protecting the healthy cells from the attack of NK cells [75].

CD96 is an inhibitory protein found on NK cells. CD96 on the surface of NK cells can binds with its ligand CD155, which may activate PI3K/AKT and other signaling pathways to downregulate the growth and activity of NK cells, as well as its the secretion of cytotoxic factors (e.g., IFN-γ), resulting in compromised anti-tumor or anti-bacterial capabilities [93-95].

In addition to inhibit the activity of NK cells, CD96 can also promote the attachment ability of NK cells, which may facilitate the adhesion of NK cells to target cells expressing CD155. Meanwhile, CD96 can interact with CD155 to enhance surface molecule exchange between NK and target cells [96].

1.4.4.2 CD96 regulates $CD8^+$ T cells

Compared to studies focusing on the regulatory role of CD96 in NK cells, there is a few research about the regulation of CD96 on $CD8^+$ T cells. The intracellular region of CD96 contains the inhibitory motif ITIM. After the phosphorylation of ITIM, it can downregulate the activity of T cells by recruiting tyrosine phosphatases that inactivate relevant signaling molecules [97]. The binding of CD96 to CD155 can inactivate signaling pathways such as PI3K/AKT, which may diminish their cytotoxic factors such as IFN-γ by suppressing the growth and activity of $CD8^+$ T cells [98-99]. It has been reported that CD96 antibodies could effectively prevent the development of colon cancer, fibrosarcoma, and melanoma in subcutaneous mice models, which was independent of NK cells, but relied on $CD8^+$ T cells [100]. CD96 also contains a stimulatory motif of YXXM. After the binding of CD96 to CD155, phosphorylation of the YXXM motif can activate downstream signaling molecules, thereby promoting, and activating $CD8^+$ T cells [63]. Overall, like TIGIT, CD96 may act as an inhibitory molecule to regulate $CD8^+$ T cells. Considering the current controversies in relevant studies, further in-depth studies are necessitated to decipher the regulatory role of CD96 in $CD8^+$ T lymphocytes.

1.5 Respiration in immune cells

The respiratory capacity of immune cells, or their metabolic activity,

especially their methods of energy production and utilization, remains a key participant in the response of the immune system [101]. Mitochondrial respiration and glycolytic pathways are two major types related to the respiratory capacity of immune cells. To be specific, mitochondrial respiration is defined as the performance of aerobic respiration by immune cells using mitochondria, which may generate a large amount of ATP through the tricarboxylic acid cycle and the electron transport chain [102]. Upon activation, different types of immune cells rely on mitochondrial respiration to varying degrees. For instance, mitochondrial oxidative phosphorylation can meet the basal metabolic needs of lymphocytes in a resting state primarily. Activated lymphocytes also require supplementary energy generated by glycolysis, in addition to energy from mitochondrial respiration [103].

A large amount of ATP produced by respiratory metabolism may satisfy the energy demands of immune cells for exerting their stimulation, proliferation, and effector functions. It also facilitates the synthesis and secretion of a substantial number of cytotoxic factors, such as IFN-γ, TNF-α [104]. Factors produced during the respiratory metabolism of immune cells can also enhance their secretion of cytotoxic factors. For instance, reactive oxygen species generated during mitochondrial respiration can act as signaling molecules to promote the activation and cytotoxic capabilities of immune cells, thereby encouraging their secretion of more cytotoxic factors [105].

Immune checkpoints including PD-1, CTLA-4, and CD96 may decrease the metabolic function of immune cells, particularly glycolysis and oxidative phosphorylation. It may further weaken their energy supply and metabolic intermediate production, resulting in reduced synthesis and secretion of cytotoxic factors [106]. It can be interpreted that immune checkpoints can further reduce the effector functions of immune cells by regulating their metabolic state, besides inhibiting immune cell activity directly via signal transduction. This understanding may provide a theoretical basis of the use of immune checkpoint blockers in immunotherapy for tumors.

1.6 Objectives of the study

The present study aims primarily to examine the possible therapeutic function of the immune checkpoint receptor CD96 in the treatment of pancreatic cancer. For this purpose, this study intended to analyze the expression level of CD96 on $CD8^+$ T cells, as well as its ligand, CD155, on pancreatic cancer cells. Our subsequent experiments woud focuse on the activation and inhibition of CD96/CD155 axis to observe changes in IFN-γ and respiration ability.

2. Materials and Methods

2.1 Materials

2.1.1 Consumables

Consumables	Company of source
6-well plates	Thermo Fisher Scientific, Roskilde, Denmark
12-well plates	Thermo Fisher Scientific, Roskilde, Denmark
96-well plates	Thermo Fisher Scientific, Roskilde, Denmark
50 ml pipette	Costar, Maine, USA
25 ml pipette	Costar, Maine, USA
10 ml pipette	Costar, Maine, USA
5 ml pipette	Costar, Maine, USA
1.5 ul pipette tips	Eppendorf, Hamburg, Germany
10 ul pipette tips	Eppendorf, Hamburg, Germany
100 ul pipette tips	Eppendorf, Hamburg, Germany
1000 ul pipette tips	Eppendorf, Hamburg, Germany
2.0 ml tips	Eppendorf, Hamburg, Germany
1.5 ml tips	Eppendorf, Hamburg, Germany
50 ml tube	Falcon, Reynosa, Mexico
15 ml tube	Falcon, Reynosa, Mexico
7.5 ml Heparin Vacuum Blood Collection	Sarstedt, Nümbrecht, Germany
Cell culture flask (T75)	Thermo Fisher Scientific, Roskilde, Denmark
Coverslips	Paul Marienfeld GmbH & Co.KG, Lauda-Königshofen, Germany
Cryotubes	Thermo Fisher Scientific, Roskilde, Denmark
Fluorescence Activated Cell Sorting (FACS) tubes	Falcon, New York, USA
Gloves	SHIELD Scientific B.V., Bennekom, Netherlands

Injector	Sarstedt, Nümbrecht, Germany
Microscope slides	Thermo Fisher Scientific, Roskilde, Denmark
Needle	Sarstedt, Nümbrecht, Germany

2.1.2 Chemicals

Chemicals	**Company or source**	**identifier**
Acetone	Sigma-Aldrich, Steinheim, Germany	67-64-1
Agilent Seahorse XFp FluxPak	Agilent technologies, California, USA	103022-100
Aqueous mounting agent	Sigma-Aldrich, Steinheim, Germany	108562
BD GolgiStop™ Protein Transport Inhibitor	BD Biosciences, New Jersey, USA	554724
BD Pharmingen™ Leukocyte Activation Cocktail	BD Biosciences, New Jersey, USA	550583
Biocoll medium	Bio&SELL, Nuremberg, Germany	BS. L6115
BLOXALL	Vector California, California, USA	SP-6000
BSA	Sigma-Aldrich, Steinheim, Germany	A9418
CD155 recombinant protine	R&D systems, Minneapolis, USA	9174-CD-050
Chloroform	Sigma-Aldrich, Steinheim, Germany	319988
Corning Cell Tak	Corning, NewYork, USA	354240
DMEM	Gibco, New York, USA	41966-029
Ethonal	PanReac AppliChem, Darmstadt, Germany	131086

FBS	Sigma-Aldrich, Steinheim, Germany	35079017
Fixation buffer	Invitrogen, Waltham, USA	00-8222-49
Hematoxylin	Sigma-Aldrich, Steinheim, Germany	MHS16
Isopropanol	Sigma-Aldrich, Steinheim, Germany	278475
Levamisole	Vector California, California, USA	SP-5000
Natriumacid	Sigma-Aldrich, Steinheim, Germany	S2002
PBS	PAN-Biotech, Munich, Germany	P04-36500
Penicillin-Streptomycin Solution	PAN-Biotech, Munich, Germany	P06-07100
Permeabilization buffer	Invitrogen, Waltham, USA	00-8333-56
Poly-D-Lysin	Thermo Fisher Scientific, Roskilde, Denmark	A3890401
RNase-free water	Qiagen, Hilden, Germany	129112
RPMI 1640 Medium	Gibco, New York, USA	21875-034
Seahorse XF 1.0M Glucose Solution	Agilent technologies, California, USA	103577-100
Seahorse XF 100mM Pyruvate Solution	Agilent technologies, California, USA	103578-100
Seahorse XF 200mM Glutamine Solution	Agilent technologies, California, USA	103579-100
Seahorse XF Calibrant Solution	Agilent technologies, California, USA	100840-000
Seahorse XF RPMI Medium	Agilent technologies, California, USA	103681-100
TBS	Sigma-Aldrich, Steinheim, Germany	T5912

Trizol	Thermo Fisher Scientific, Roskilde, Denmark	15596018
Trypsin/EDTA	Lonza, St. Louis, USA	BE17-161E
Tween-20	Sigma-Aldrich, Steinheim, Germany	9005-64-5

2.1.3 Antibodies

Antibodies	**Fluorochrome**	**Company of source**	**Identifier**
CD155 (Isotype: Mouse IgG2a)	Alexa Fluor 647	BD Biosciences, New Jersey, USA	566305
Isotype control (Mouse IgG2a)	Alexa Fluor 647	BD Biosciences, New Jersey, USA	565357
CD96	-	Abcam, Cambridge, UK	AB81717
CD96	BV421	BD Biosciences, New Jersey, USA	742794
CD4	BUV395	BD Biosciences, New Jersey, USA	563550
CD45	BV650	BD Biosciences, New Jersey, USA	563717
CD3	PerCP Cy5.5	BD Biosciences, New Jersey, USA	300430
CD8	APC-H7	BD Biosciences, New Jersey, USA	560179
IFN-γ	FITC	BD Biosciences, New Jersey, USA	552882
CD155 (Isotype: Mouse IgG1)	-	Thermo Fisher Scientific, Roskilde, Denmark	MA5-13493
Isotype control (Mouse IgG1)	-	Abcam, Cambridge, UK	ab91353
Biotinylated Horse Anti-Mouse IgG	-	Thermo Fisher Scientific, Roskilde, Denmark	31806

2.1.4 Primers

Primers	Company of source	Identifier
CD155	OriGene, Herford, Germany	HP209435
GAPDH	OriGene, Herford, Germany	HP205798

2.1.5 Commercial assays kits

Product	Company or source	Identifier
Avidin/Biotin Blocking Kit	Vector California, California, USA	SP-2001
Dynabeads™ Human T-Activator CD3/CD28 for Expansion and Activation of T Cells	Thermo Fisher Scientific, Roskilde, Denmark	11161D
QuantiNova SYBR Green PCR Kit	Qiagen, Hilden, Germany	208056
QuantiTect Reverse Transcription Kit	Qiagen, Hilden, Germany	205311
Seahorse XFp Real-Time ATP Rate Assay Kit	Agilent technologies, California, USA	103591-100
VECTASTAIN® ABC-AP Kit, Alkaline Phosphatase (Standard)	Vector California, California, USA	AK-5000
Vector® Red Substrate Kit, Alkaline Phosphatase (AP)	Vector California, California, USA	SK-5100

2.1.6 Apparatus

Apparatus	Company of source
Autoclave	Unisteri, Oberschleißheim, Germany
Bio-Rad CFX96 Real-Time PCR system	Bio-Rad Laboratories, California, USA

Centrifuge	Hettich, Ebersberg, Germany
ChemiDoc Imaging System	Bio-Rad Laboratories, California, USA
CO_2 Incubator	Binder, Tuttlingen, Germany
Cool Centrifuge	Eppendorf, Hamburg, Germany
DNA workstation	Uni Equip, Martinsried, Germany
Drying cabinet	Thermo Fisher Scientific, Schwerte, Germany
Electronic pH meter	Knick Elektronische Messgeräte, Berlin, Germany
FACS Fortessa	BD Biosciences, Heidelberg, Germany
Fridge (4°C, -20°C and -80°C)	Siemens, Munich, Germany
Ice machine	KBS, Mainz, Germany
Inverted light microscope	Nikon, Tokio, Japan
Lamina flow	Thermo Fisher Scientific, Schwerte, Germany
Liquid Nitrogen tank	MVE Goch, Germany
Magnet separator	Thermo Fisher Scientific, Schwerte, Germany
Micro centrifuge	Labtech, Ebersberg, Germany
Micro weigh	Micro Precision Calibration, California, USA
Microscope	Olympus, Hamburg, Germany
Pipette boy	Eppendorf, Hamburg, Germany
Seahorse XFp Analyzer	Agilment, California, USA
Shaker	Edmund Bühler, Bodelshausen, Germany
Thermocycler	Eppendorf, Hamburg, Germany
Thermomixer comfort	Eppendorf, Hamburg, Germany
Water bath	Memmert, Schwabach, Germany

2.1.7 Software

Software and version	Company
FlowJo Vesion 10.0	BD Biosciences, Heidelberg, Germany
Graphpad Prism 7.04	GraphPad, Boston, USA
Image-Pro Plus 6.0	Media Cybernetics, Maryland, USA
Wave	Agilent technologies, California, USA

2.1.8 Buffer and solutions

FACS buffer

BSA	5 g
DPBS	1 L
Natriumacid	2 ml

TBST

TBS	10× TBS diluted with double distilled water
Tween-20	0.1%

Seahorse assay medium

Seahorse XF RPMI Medium	9.7 ml
Seahorse XF 200mM Glutamine Solution	100 µl
Seahorse XF 100mM Pyruvate Solution	100 µl
Seahorse XF 1.0M Glucose Solution	100 µl

2.2 Methods

2.2.1 Cell culture

The PANC-1, AsPC-1, PSN-1, and MiaPaCa-2 pancreatic cancer cell lines were bought from ATCC (Manassas, Virginia, USA) and kept in nitrogen-filled reservoirs at the laboratory of department of General, Visceral, and Transplantation Surgery of Ludwig Maximilian University. PANC-1 and MiaPaCa-2 were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin, while AsPC-1 and PSN-1 were cultured in RPMI 1640 supplemented with 10% FBS and 1% penicillin-streptomycin. Regular mycoplasma infection screenings were conducted in our laboratory. These cell lines were kept inside T75 flasks for culture and incubated in an incubator with humidity at 37°C and 5% CO_2. The culture media was passed and refreshed every three days.

2.2.2 RNA isolation, reverse transcription and quantitative polymerase chain reaction (qPCR)

RNA isolation:

To lyse cells, the culture medium was removed, and 1 ml of TRIzol™ Reagent was added per 1×10^5-1×10^7 cells in a T75 culture flask. The lysate was pipetted upward and downward several times to homogenize it. After incubating for 5 minutes, the lysate was transferred to a new tube. 0.2 ml chloroform per 1 ml TRIzol™ Reagent was added to the nucleoprotein complexes to separate into three different phases. The tube was covered and shaken vigorously, then placed for 3 minutes. The tube was centrifuged at 12,000 ×g for 15 minutes at 4°C. The mixture was divided into three distinct phases: a lower phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. The aqueous phase carrying the RNA was transferred to a fresh tube. 0.5 ml isopropanol per 1 ml of TRIzol™ reagent was added into the aqueous phase to precipitate RNA. After that, the fresh tube was placed around 10 minutes at 4℃ and spined for 10 minutes at 12,000 ×g during 4°C, then the supernatant was removed with a micro pipettor. To wash

the pellet, it was resuspended in 1 ml of 75% ethanol per 1 ml of TRIzol™ Reagent. The mixture was vortexed briefly and centrifuged at 7,500 ×g for 5 minutes at 4°C. The supernatant was removed, and the RNA pellet was air-dried for approximately 10 minutes. To resuspend the pellet, it was pipetted up and down in 50 µl of RNase-free water. The mixture was incubated in a water bath at 55-60°C for 10 minutes. The RNA was either used immediately for downstream applications or stored at -80°C.

Reverse transcription:

Following RNA isolation and reverse transcription procedure were performed to synthesize the RNA into cDNA according to the QuantiTect® Reverse Transcription Kit. First, the genomic DNA elimination step was performed on ice according to Table 1. The mixture was vortexed thoroughly, incubated in the thermocycler at 42°C for 2 minutes, and then immediately placed on ice.

For the reverse transcription, the primers sequences are listed in Table 2. The primers were of commercial origin and therefore went all quality control procedures as described in the datasheet. The reverse-transcription master mix was prepared on ice according to the instructions in Table 3. Then the program in Thermocycler was set as: 42℃ 15 minutes, 95℃ 3 minutes. The reverse-transcription reactions were put into Thermocycler, then the program was started. The cDNA products were either used immediately for qPCR or stored at -20°C for long-term storage.

Component	Volume/reaction
RNase-free water	Variable
Template RNA, up to 1 µg	Variable
gDNA Wipeout Buffer, 7x	2 µl
Total reaction volume	14 µl

Table 1: Genomic DNA removal reaction components

CD155	Forward sequence	CACTGTCACCAGCCTCTGGATA
	Reverse sequence	TCATAGCCAGAGATGGATACCTC
GAPDH	Forward sequence	GTCTCCTCTGACTTCAACAGCG
	Reverse sequence	ACCACCCTGTTGCTGTAGCCAA

Table 2: Primer sequnce

Component	Volume/reaction
Entire genomic DNA elimination reaction	14 µl
Quantiscript RT Buffer, 5x	4 µl
Quantiscript Reverse Transcriptase	1 µl
Primer	1 µl
Total reaction volume	20 µl

Table 3: Reverse-transcription process components

qPCR:

After reverse transcription, qPCR was performed to measure the expression of CD155 in four pancreatic cancer cell lines mentioned before using the QuantiNova SYBR Green PCR Kit. The sample without cDNA was used as a negative technical control. The housekeeping gene GAPDH served as a positive control. The gene expression levels of CD155 in various cell lines were calculated using the $2^{-\Delta CT}$ values, $\Delta CT = CT_{CD155} - CT_{GAPDH}$. Each sample underwent independent triplicate experiments.

The reaction mixture was prepared according to the protocol in Table 4. The mixture was vortexed thoroughly before being transferred to qPCR tubes. Cycling condition is shown in Table 5.

Component	96-well block, Rotor-Gene	Final concentration
QN ROX Reference Dye	2 µl	1x
2x SYBR Green PCR Master Mix	10 µl	1x
Primer A	Variable	0.7 µM
Primer B	Variable	0.7 µM
Template cDNA	Variable	≤100 ng/reaction
RNase-free water	Variable	-
Total reaction volume	20 µl	-

Table 4: Setup of QuantiNovaTM SYBR Green PCR Kit

Step	Time	Temperature	Ramp rate
PCR initial heat activation	2 minutes	95°C	Maximal/fast mode
Denaturation	5 seconds	95°C	Maximal/fast mode
Combined annealing/extension	5 seconds	60°C	Maximal/fast mode
Number of cycles	40	-	-

Table 5: Cycling conditions of QuantiNovaTM SYBR Green PCR Kit

2.2.3 Immunocytochemistry (ICC)

To be sure that CD155 is also expressed at the protein level. ICC experiments with four pancreatic cancer cell lines mentioned before were conducted:

Day 1: For sterilization, cover slips were soaked in 100% alcohol for 10 minutes, then washed three times with PBS. Then cover slips were coated with Poly-D-Lysine for 5 minutes. The cover slips were placed in 6-well plates and exposed to UV light for 30 minutes. $5x10^5$ pancreatic cancer cells were seeded into each well. Finally, culture medium was added to each well and the plates were incubated in an incubator with humidity at 37°C and 5% CO_2.

Day 2: The culture medium was discarded from the 6-well plates and the plates were gently washed three times with PBS. Acetone (pre-cooled at -20°C) was added to fix the cells, and the plates were placed at -20°C for 10 minutes. The plates were then washed with TBST for 5 minutes, and this step was repeated three times. For blocking non-specific binding, the ready-to-use Avidin solution and Biotin solution were added into the 6-well plates for 20 minutes at room temperature. Then the plates were washed with TBST for 5 minutes and incubated in a mixture of PBS and 3% BSA for 1 hour. CD155 antibodies (1:50 dilution) or isotype control antibodies (1:50 dilution) were added into the plates, then the plates were incubated overnight at 4°C.

Day 3: The antibodies were discarded from the 6-well plates and then the plates were gently washed with TBST for 5 minutes, this step was repeated for three times. Biotinylated Horse Anti-Mouse IgG (1:200 dilution) was added into the 6-well plates for 30 minutes at room temperature. The 6-well plates were washed with TBS for 5 minutes, this step was repeated for three times. The working solution from the ABC-AP Kit was added into the plates, then the plates were placed for 30 minutes at room temperature. The 6-well plates were washed with TBS for 5 minutes, this step was repeated for three times. After that, the working solution from the Vector Red Substrate Kit and Levamisole was added to the plates, then the plates were incubated in a humidified chamber for 20 minutes at room temperature. After that, the

plates were washed with PBS for 5 minutes and then were gently rinsed with distilled water. After that, the cover slips were briefly put in hematoxylin solution for 1 second and then rinsed in tap water for 5 minutes. The plates were observed under a microscope, air-dried completely, and sealed with neutral resin.

Image-Pro Plus is a sophisticated image analysis software developed by Media Cybernetics. It is widely used in research and industrial applications for advanced image processing and quantitative analysis. This software was used by us to quantify the CD155-positive index in the immunocytochemical images. First, the intensity calibration was made, then magenta points which stand for the CD155-positive index were chosen to the points we are interested in, and the Image-Pro Plus will automatically identify all the magenta area. Then the IOD (integrated optical density) were measured. The mean intensity was calculated as the IOD divided by the photo area.

2.2.4 Peripheral blood mononuclear cells (PBMCs) isolation

The blood samples were collected from healthy donors. At the time of blood collection, the donors had no signs of acute febrile illness. The blood collection was approved by ethical committee (#02512), and signed informed consents were collected.

For the PBMCs isolation, Biocoll medium was pipetted into a 50 ml Falcon tube, whole blood and PBS were mixed in a 1:1 ratio to dilute the blood. The PBS-diluted blood was carefully layered on top of the Biocoll medium in a 1:1 ratio, ensuring minimal mixing between the two layers (set the pipetting speed to the lowest level). The tube was centrifuged with 800 ×g/20 minutes/20°C (without break). PBMCs were removed carefully without sucking up Biocoll medium, then the PBMCs were moved into a fresh 50 ml Falcon tube. At least 3 volumes of PBS were added to the PBMCs, then the tube was centrifuged with 300 ×g/10 minutes/ 20°C (with break), PBS was used to resuspend the pellet after discarding the supernatant, then the tube was centrifuged with 300 ×g/10 minutes/ 20°C (with break). Finally, the PBMCs were resuspended in medium (RPMI + 10% FBS + 1% penicillin-

streptomycin) after discarding the supernatant for cell counting.

The process of isolating PBMCs can be shown in Figure 5.

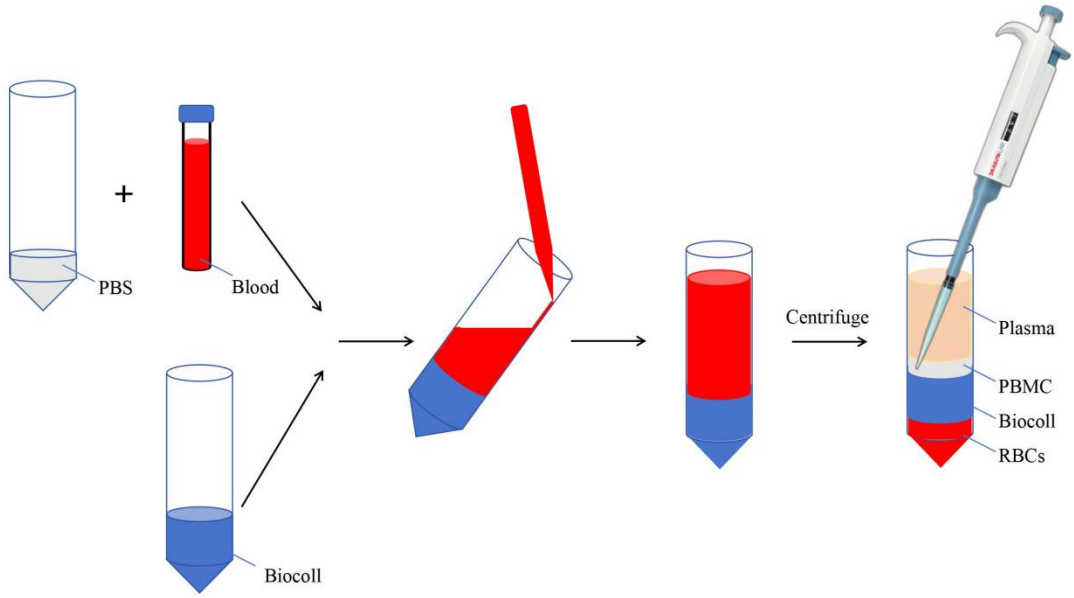

Figure 5: The process of isolating PBMCs

2.2.5 PBMCs stimulation

For PBMCs stimulation, two methods were applied (Figure 6). First, Leukocyte Activation Cocktail was used to stimulate PBMCs while measuring FACS analysis.

PBMCs were resuspended in medium (RPMI + 10% FBS + 1% penicillin-streptomycin) for cell counting. After counting cells, PBMCs were diluted into $1x10^6$ cells/ml in the medium. 1 ml PBMCs suspension was transferred into a 15 ml Falcon tube. To stimulate the PBMCs, 2 µl Leukocyte Activation Cocktail was added to each tube. At the same time, CD155 recombinant protine (5 µg/ml, 10 µg/ml, 20 µg/ml) and CD96 antibodies (5 µg/ml) were added to the treatment groups. The tubes were not closed, the lids were placed loosely on the tubes to allow gas exchange. The tubes were incubated in incubator with humidity at 37°C and 5% CO_2 for 4 hours. 10 ml FACS buffer was added into 15 ml Falcon tubes to wash PBMCs, supernatant was discarded after spinning with 300 ×g/5 minutes/20°C, this step was repeated

for two times. Finally, 100 µl FACS buffer was added into the 15 ml Falcon tube to resuspend PBMCs, then PBMCs were removed to FACS tube. The PBMCs suspension was ready to do staining for FACS analysis.

For measuring mitochondrial metabolism, Dynabeads™ Human T-Activator CD3/CD28 was used to stimulate PBMCs.

PBMCs were resuspended in medium (RPMI + 10% FBS + 1% penicillin-streptomycin) for cell counting. After counting cells, PBMCs were diluted to $1x10^6$ cells/ml in the medium. 1 ml of the suspension was added to 15 ml Falcon tubes. 25 µl pre-washed and resuspended Dynabeads were then added to each tube. At the same time, CD155 recombinant protein (5 µg/ml) and CD96 antibodies (5 µg/ml) were added to the treatment groups. The tubes were incubated in incubator with humidity at 37°C and 5% CO_2 for 48 hours. The 15 ml Falcon tubes were not closed, the lids were placed loosely on the tubes to allow gas exchange. The stimulated PBMCs were collected, the beads were removed with a magnet separator. The stimulated PBMCs were used directly to measure mitochondrial metabolism.

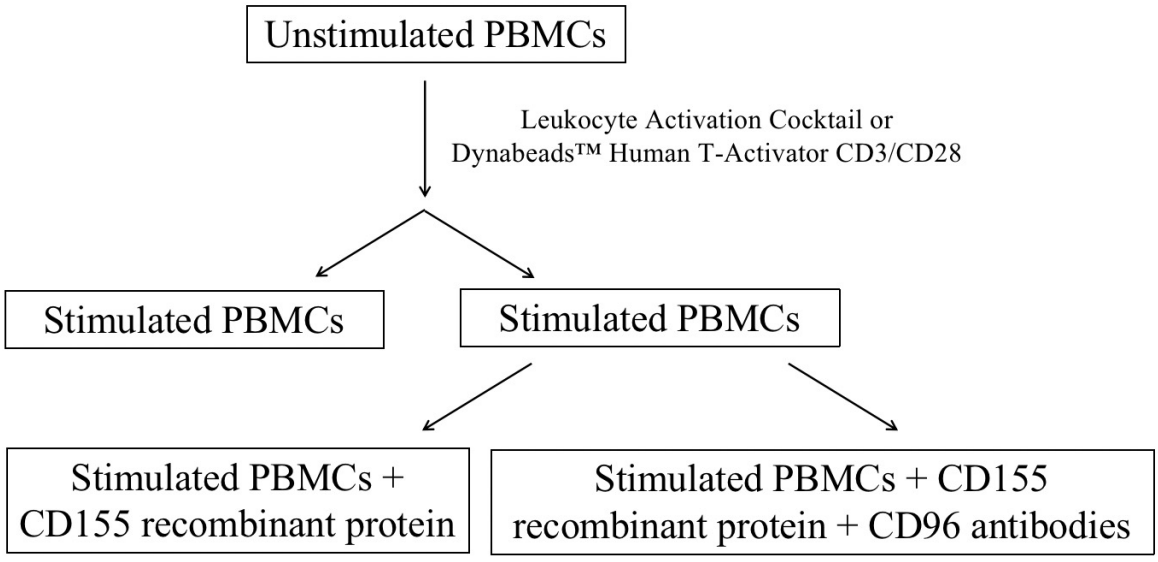

Figure 6: The schema of PBMCs stimulation

2.2.6 FACS measurement

For extracellular staining, FACS tubes were filled with 100 µl PBMCs suspension (1x10^6 cells). Extracellular antibodies were added to the FACS tubes and were incubated for 30 minutes protected from light at room temperature. 2 ml FACS buffer was added to each FACS tube, then centrifuged at 500 ×g for 5 minutes at room temperature, then supernatant was discarded. 200-500 µl FACS buffer was added into each FACS tube, then the PBMCs were ready to be measured.

For the simultaneous extracellular and intracellular staining, FACS tubes were filled with 100 µl PBMCs suspension (1x10^6 cells). Extracellular antibodies were added in the FACS tubes and were incubated for 30 minutes under the shadows at room temperature. Each FACS tube was filled with 100 µl fixation buffer, and then was placed for 20 minutes in darkness at room temperature. 2 ml 1x permeabilization buffer was added in each FACS tube and the FACS tube were spined with 500 ×g at room temperature for 5 minutes, then the supernatant was discarded, this step was repeated two times. Intracellular antibodies were added and incubated for 30-60 minutes in darkness at room temperature. 2 ml 1x permeabilization buffer was added in each FACS tube, and then the FACS tubes were spined with 500 ×g at room temperature for 5 minutes, then the supernatant was discarded. 2 ml FACS buffer was added into each FACS tube, the tubes were spined with 500 ×g at room temperature for 5 minutes, then the supernatant was discarded. Each FACS tube was resuspended in 200-500 µl FACS buffer, then the PBMCs is ready to be measured.

The following tables are panels designed for different FACS experiments.

CD96 Panel	BUV395	BV650	APC-H7	BV421
Unstained[1]	-	-	-	-
FMO stim.[2]	CD4	CD45	CD8	-
Stim.[3]	CD4	CD45	CD8	CD96

Table 6: CD96 Panel: Measuring CD96 expression in $CD8^+$ T cells

[1] Unstained: PBMCs without any antibody staining.

[2] FMO stim.: Stimulated PBMCs stained with all antibodies except anti-CD96 antibodies.

[3] Stim.: Stimulated PBMCs stained with all antibodies.

- means no antibody was added.

CD155 Panel	APC
Isotype control	-
Extra. Staining[1]	CD155

Table 7: CD155 Panel: Measuring CD155 expression on pancreatic cancer cells

Isotype control: Pancreatic cancer cells stained with isotype control antibodies.

[1] Extra. staining: Extracellular staining.

- means no specific binding with CD155.

IFN-γ Panel 1	BUV395	BV650	PerCP Cy5.5	FITC	APC-H7	BV421
Unstained[1]	-	-	-	-	-	-
FMO 1 stim.[2]	CD4	CD45	CD3	-	CD8	CD96
FMO 2 stim. + CD155 5 µg/ml[3]	CD4	CD45	CD3	-	CD8	CD96
FMO 3 stim. + CD155 10 µg/ml[4]	CD4	CD45	CD3	-	CD8	CD96
FMO 4 stim. + CD155 20 µg/ml[5]	CD4	CD45	CD3	-	CD8	CD96
FMO 5 stim.[6]	CD4	CD45	CD3	IFN-γ	CD8	-
FMO 6 stim. + CD155 5 µg/ml[7]	CD4	CD45	CD3	IFN-γ	CD8	-
FMO 7 stim. + CD155 10 µg/ml[8]	CD4	CD45	CD3	IFN-γ	CD8	-
FMO 8 stim. + CD155 20 µg/ml[9]	CD4	CD45	CD3	IFN-γ	CD8	-
FMO 9 unstim.[10]	CD4	CD45	CD3	IFN-γ	CD8	-

Unstim.[11]	CD4	CD45	CD3	IFN-γ	CD8	CD96
Stim.[12]	CD4	CD45	CD3	IFN-γ	CD8	CD96
Stim. + CD155 5 μg/ml[13]	CD4	CD45	CD3	IFN-γ	CD8	CD96
Stim. + CD155 10 μg/ml[14]	CD4	CD45	CD3	IFN-γ	CD8	CD96
Stim. + CD155 20 μg/ml[15]	CD4	CD45	CD3	IFN-γ	CD8	CD96

Table 8: IFN-γ Panel 1: Measuring IFN-γ expression when culture PBMCs with different concentration of CD155 recombinant protein

[1] Unstained: PBMCs without any antibody staining.

[2] FMO 1 stim.: Stimulated PBMCs stained with all antibodies except anti-IFN-γ antibodies.

[3] FMO 2 stim. + CD155 5 μg/ml: Stimulated PBMCs cultured with 5 μg/ml CD155 recombinant protein stained with all antibodies except anti-IFN-γ antibodies.

[4] FMO 3 stim. + CD155 10 μg/ml: Stimulated PBMCs cultured with 10 μg/ml CD155 recombinant protein stained with all antibodies except anti-IFN-γ antibodies.

[5] FMO 4 stim. + CD155 20 μg/ml: Stimulated PBMCs cultured with 20 μg/ml CD155 recombinant protein stained with all antibodies except anti-IFN-γ antibodies.

[6] FMO 5 stim.: Stimulated PBMCs stained with all antibodies except anti-CD96 antibodies.

[7] FMO 6 stim. + CD155 5 μg/ml: Stimulated PBMCs cultured with 5 μg/ml CD155 recombinant protein stained with all antibodies except anti-CD96 antibodies.

[8] FMO 7 stim. + CD155 10 μg/ml: Stimulated PBMCs cultured with 10 μg/ml CD155 recombinant protein stained with all antibodies except anti-CD96 antibodies.

[9] FMO 8 stim. + CD155 20 μg/ml: Stimulated PBMCs cultured with 20 μg/ml

CD155 recombinant protein stained with all antibodies except anti-CD96 antibodies.

[10] FMO 9 unstim.: Unstimulated PBMCs stained with all antibodies except anti-CD96 antibodies.

[11] Unstim.: Unstimulated PBMCs stained with all antibodies.

[12] Stim.: Stimulated PBMCs stained with all antibodies.

[13] Stim. + CD155 5 µg/ml: Stimulated PBMCs cultured with 5 µg/ml CD155 recombinant protein stained with all antibodies.

[14] Stim. + CD155 10 µg/ml: Stimulated PBMCs cultured with 10 µg/ml CD155 recombinant protein stained with all antibodies.

[15] Stim. + CD155 20 µg/ml: Stimulated PBMCs cultured with 20 µg/ml CD155 recombinant protein stained with all antibodies.

- means no antibody was added.

IFN-γ Panel 2	BUV395	BV650	PerCP Cy5.5	FITC	APC-H7	BV421
Unstained[1]	-	-	-	-	-	-
FMO 1 stim.[2]	CD4	CD45	CD3	-	CD8	CD96
FMO 2 stim. + CD155 5 µg/ml[3]	CD4	CD45	CD3	-	CD8	CD96
FMO 3 stim. + CD155 5 µg/ml + CD96 Ab. 5 µg/ml[4]	CD4	CD45	CD3	-	CD8	CD96
FMO 4 stim.[5]	CD4	CD45	CD3	IFN-γ	CD8	-
FMO 5 stim. + CD155 5 µg/ml[6]	CD4	CD45	CD3	IFN-γ	CD8	-
FMO 6 stim. + CD155 5 µg/ml + CD96 Ab. 5 µg/ml[7]	CD4	CD45	CD3	IFN-γ	CD8	-
FMO 7 unstim.[8]	CD4	CD45	CD3	IFN-γ	CD8	-
Unstim.[9]	CD4	CD45	CD3	IFN-γ	CD8	CD96
Stim.[10]	CD4	CD45	CD3	IFN-γ	CD8	CD96

Stim. + CD155 5 μg/ml[11]	CD4	CD45	CD3	IFN-γ	CD8	CD96
Stim. + CD155 5 μg/ml + CD96 Ab. 5 μg/ml[12]	CD4	CD45	CD3	IFN-γ	CD8	CD96

Table 9: IFN-γ Panel 2: Measuring IFN-γ expression when culture PBMCs with CD155 recombinant protein and CD96 antibodies

[1] Unstained: PBMCs without any antibody staining.

[2] FMO 1 stim.: Stimulated PBMCs stained with all antibodies except anti-IFN-γ antibodies.

[3] FMO 2 stim. + CD155 5 μg/ml: Stimulated PBMCs cultured with 5 μg/ml CD155 recombinant protein stained with all antibodies except anti-IFN-γ antibodies.

[4] FMO 3 stim. + CD155 5 μg/ml + CD96 Ab. 5 μg/ml: Stimulated PBMCs cultured with 5 μg/ml CD155 recombinant protein and 5 μg/ml CD96 antibodies stained with all antibodies except anti-IFN-γ antibodies.

[5] FMO 4 stim.: Stimulated PBMCs stained with all antibodies except anti-CD96 antibodies.

[6] FMO 5 stim. + CD155 5 μg/ml: Stimulated PBMCs cultured with 5 μg/ml CD155 recombinant protein stained with all antibodies except anti-CD96 antibodies.

[7] FMO 6 stim. + CD155 5 μg/ml + CD96 Ab. 5 μg/ml: Stimulated PBMCs cultured with 5 μg/ml CD155 recombinant protein and 5 μg/ml CD96 antibodies stained with all antibodies except anti-CD96 antibodies.

[8] FMO 7 unstim.: Unstimulated PBMCs stained with all antibodies except anti-CD96 antibodies.

[9] Unstim.: Unstimulated PBMCs stained with all antibodies.

[10] Stim.: Stimulated PBMCs stained with all antibodies.

[11] Stim. + CD155 5 μg/ml: Stimulated PBMCs cultured with 5 μg/ml CD155 recombinant protein stained with all antibodies.

[12] Stim. + CD155 5 µg/ml + CD96 Ab. 5 µg/m: Stimulated PBMCs cultured with 5 µg/ml CD155 recombinant protein and 5 µg/ml CD96 antibodies stained with all antibodies.

- means no antibody was added.

2.2.7 Mitochondrial metabolism measurement

In order to assess the mitochondrial metabolism of PBMCs under different treatment conditions, the Seahorse XFp Real-Time ATP Rate Assay Kit was used.

First, the PBMCs stimulation and treatment were performed as described in part 2.2.5. For the PBMCs are better to attach to XFp cell culture microplate, the Cell-Tak solution was diluted to 22.4 µg/ml, then 25 µl of the diluted solution was added to each well of the XFp cell culture microplate, and the microplate was left at room temperature for 20 minutes. Afterward, the microplate was washed twice with 200 µl of PBS and air-dried. The Cell-Tak-coated XFp cell culture microplates can be used directly in the following experiments or stored at 4°C for up to one week.

After that, the Agilent Seahorse XFp Analyzer was turned on and allowed to warm up overnight. The sensor cartridge was hydrated in the utility plate at 37°C in a non-CO_2 incubator overnight.

In the second day, assay medium and XF Calibrant solution were warmed at 37°C non-CO_2 incubator at least 2 hours. The water left on the utility plate was removed, each well of the utility plate was refilled with 200 µl pre-warmed XF calibrant solution and the moats around the outside of the wells were filled with 400 µl pre-warmed XF calibrant solution. The sensor cartridge was put on the utility plate and then was incubated for 45 minutes within a 37°C non-CO_2 incubator. After removing beads with a magnet separator from the stimulated PBMCs, the PBMCs (both stimulated and unstimulated) were centrifuged with 200 ×g at room temperature for 5 minutes and then resuspended in warm assay media with a concentration of $3x10^6$ PBMCs/ml. Next, 50 µl PBMCs suspension (both stimulated and

unstimulated) was added into each well of the Cell-Tak-Coated XFp cell cultivation microplate, and 50 µl assay medium was added into the background well. The Cell-Tak-Coated XFp cell cultivation microplate was centrifuged with 200 ×g (without break) for 1 minute, then carefully verifying the cells are firmly attached on the microplate's surface, then the microplate was moved to a 37°C non-CO_2 incubator for 30 minutes. After that, 130 µl warmed assay media was added into each well carefully and slowly, then the microplate was returned into the incubator for 20 minutes. Following the preparation of compound working solutions according to the instructions, the compound working solutions were put into the ports of sensor cartridge respectively (port A: Oligomycin 20 µl; port B: rotenone + antimycin A 22 µl). Finally, the assay was run using the Agilent Seahorse XFp Analyzer. Data analysis was performed with Wave desktop software.

2.2.8 Bioinformatic analysis

For bioinformatic analysis, GEPIA is a web-based platform designed to enable the interactive exploration of RNA sequencing data from TCGA and GTEx projects (http://gepia.cancer-pku.cn/index.html). With this platform, it is possible to analyze and visualize gene expression patterns in various tumor types and healthy tissues. This platform was utilized to study the expression levels of CD96 and CD155 in pancreatic cancer tissues and normal pancreatic tissues, as well as the overall survival in pancreatic cancer patients with high and low CD96 expression in pancreatic cancer tissues.

2.2.9 Statistical analysis

All experiments were conducted independently with three replicates each. The mean and standard deviation (SD) was calculated for each group. Comparisons among groups were performed using non-parametric Kruskal-Wallis test or Mann-Whitney test. Significance levels are denoted as follows: *$P<0.05$, **$P<0.01$.

3. Results

3.1 CD96 is expressed on $CD8^+$T cells

To determine whether $CD8^+$ T cells among PBMCs express CD96, fresh blood was first obtained from veins of healthy donors. PBMCs were then extracted and stimulated by Leukocyte Activation Cocktail reagent, and CD96 expression was assessed using FACS analysis. The gating strategy is as shown in *Figure 7*, $CD8^+$ T cells expressing CD96 were identified as a $CD45^+CD8^+CD96^+$ population, while the $CD4^+$ T cells expressing CD96 were identified as a $CD45^+CD4^+CD96^+$ population. The results revealed that stimulated and unstimulated $CD8^+$ T cells both express CD96. Notably, the expression of CD96 in unstimulated $CD8^+$ T cells was more pronounced compared with that in stimulated $CD8^+$ T cells. Additionally, we explored whether $CD4^+$ T cells express CD96. The findings indicated that CD96 was expressed in unstimulated $CD4^+$ T cells, but no CD96 expression was observed in stimulated $CD4^+$ T cells (*Figure 8*).

Therefore, we confirmed that CD96 can be found on $CD8^+$ T cells, providing the basis for us to use CD96 antibodies to block the interaction between CD96 which expressed on stimulated $CD8^+$ T cells and its ligand CD155 in the following experiments.

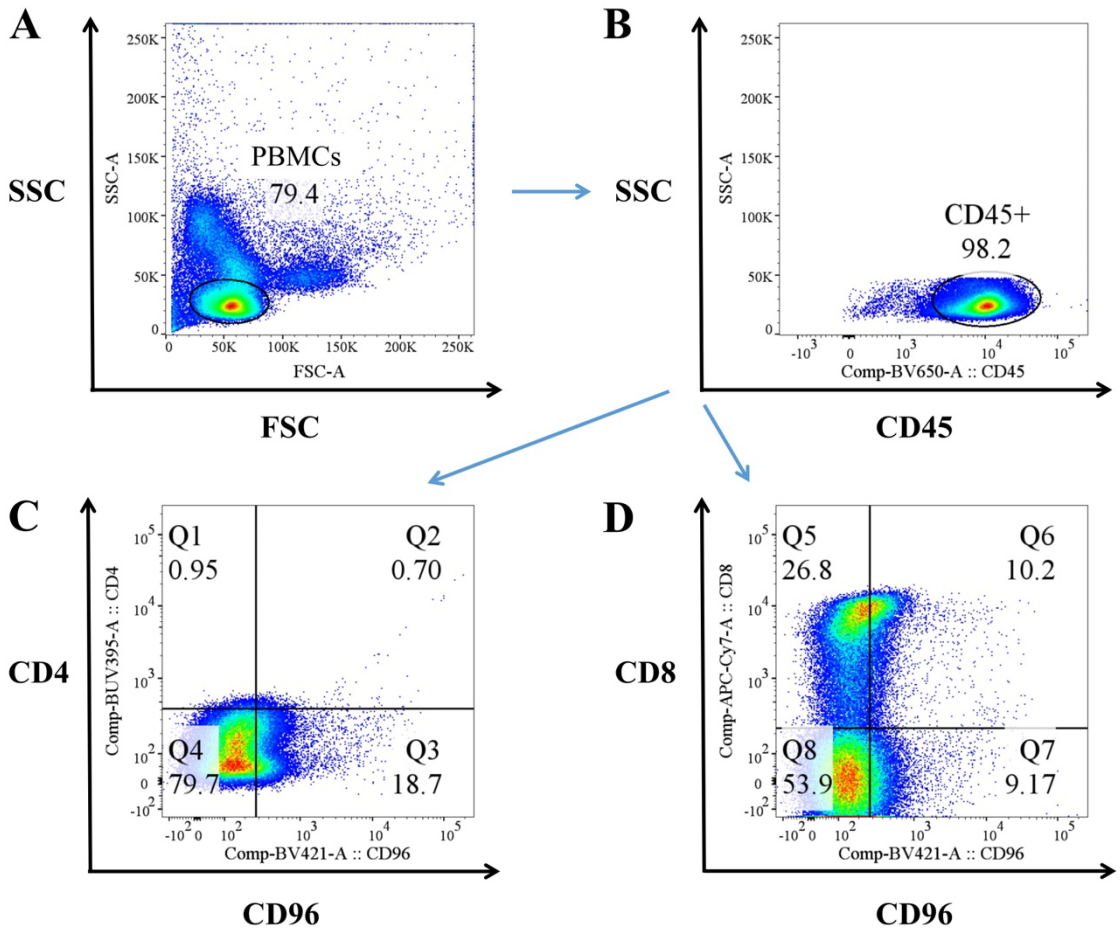

Figure 7: Gating strategy to detect CD96 activity on $CD8^+$ T cells using FACS analysis

(A) PBMCs in gate; (B) $CD45^+$ cells in gate; (C) $CD4^+CD96^+$ T cells in Q2; (D) $CD8^+CD96^+$ T cells in Q6.

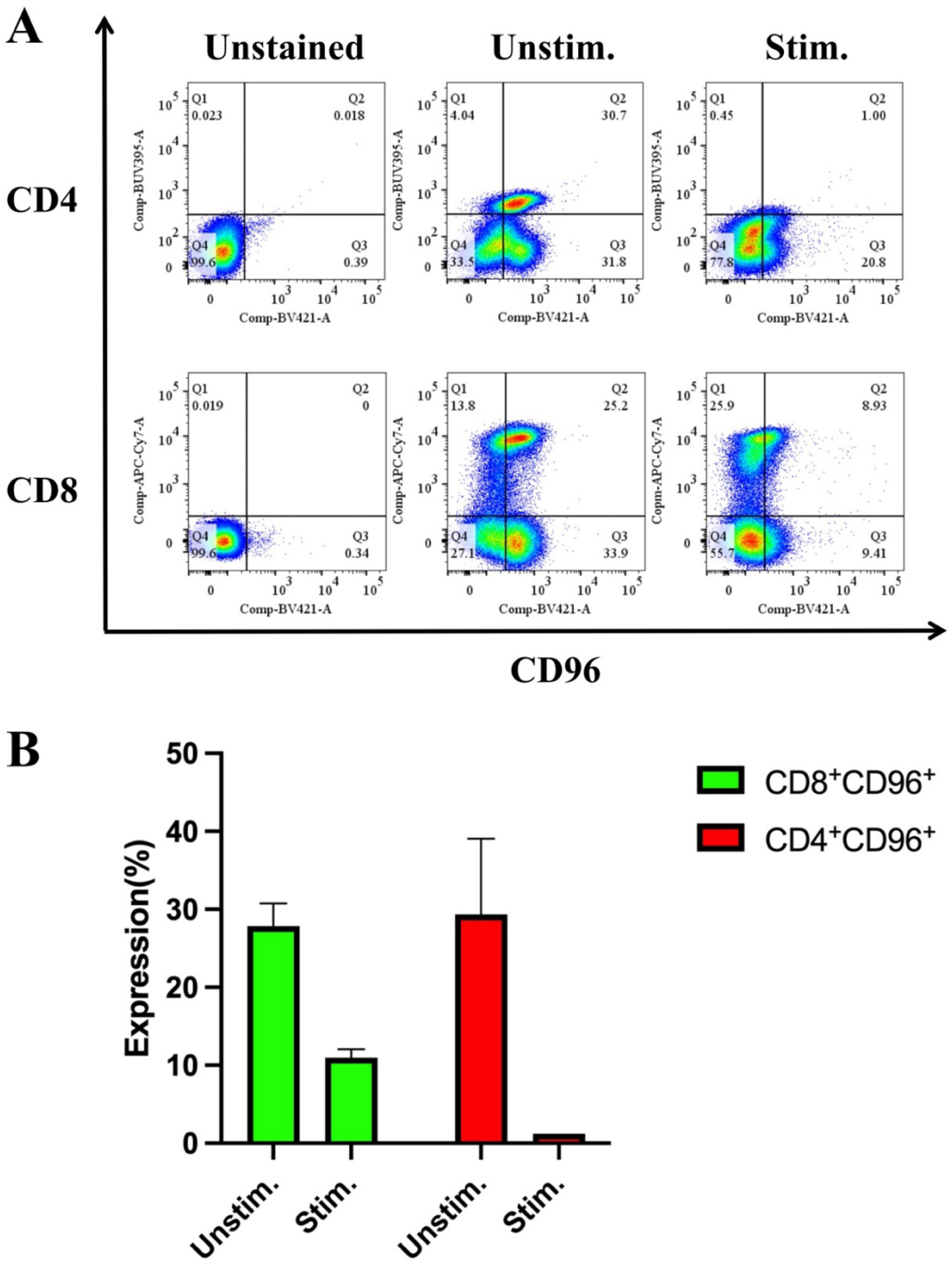

Figure 8: CD96 expression on $CD8^+$ T and $CD4^+$ T cells was measured using FACS analysis

(A) The expression of $CD8^+CD96^+$ T cells and $CD4^+CD96^+$ T cells in unstimulated and stimulated PBMCs using FACS analysis from three different blood donors; (B) Column graph of the expression of $CD8^+CD96^+$ T cells and $CD4^+CD96^+$ T cells in unstimulated and stimulated PBMCs. Three independent repeated experiments were performed. All data are

presented as mean ± SD.

Unstained means PBMCs stained without antibodies; Unstim. means Unstimulated PBMCs; Stim. means Stimulated PBMCs.

3.2 CD155 is expressed on pancreatic cancer cell lines

As mentioned in introduction part, CD155 is the major ligand of CD96. To investigate the potential efficacy of blocking CD96 for potential therapy of pancreatic cancer, it is necessary to determine whether CD155 as a protein can be found on the surface of pancreatic cancer cells. Three different methods (qPCR analysis, FACS analysis, and ICC) were employed to assess CD155 expression in the following pancreatic cell lines: PANC-1, AsPC-1, PSN-1, and MiaPaCa-2.

In qPCR analysis, varying levels of CD155 expression were observed among the four pancreatic cell lines (*Figure 9*). FACS analysis results demonstrated abundant extracellular expression of CD155 on the cells (*Figure 10*). The ICC findings revealed CD155 expression was found on the pancreatic cancer cells (*Figure 11*).

In conclusion, after measuring CD155 expression in four pancreatic cancer cell lines by qPCR analysis, FACS analysis and ICC, we confirmed that CD155 can be found on pancreatic cancer cells (*Table 10*).

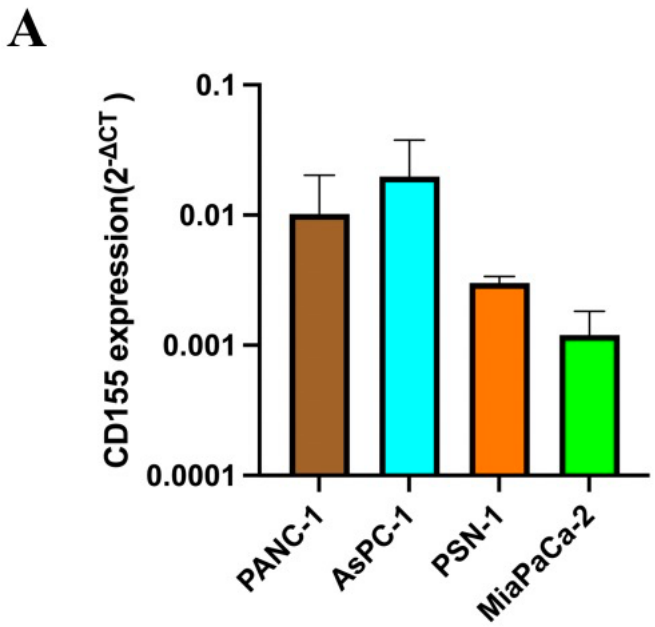

Figure 9: CD155 expression on pancreatic cancer cell lines was measured using qPCR analysis

(A) The expression level of CD155 of four pancreatic cancer cell lines expressed by the $2^{-\Delta CT}$ value, $\Delta CT = CT_{CD155} - CT_{GAPDH}$. 1×10^7 cells in each pancreatic cancer cell lines were taken to extract RNA and did the qPCR analysis. Three independent repeated experiments were performed. All data are presented as mean ± SD.

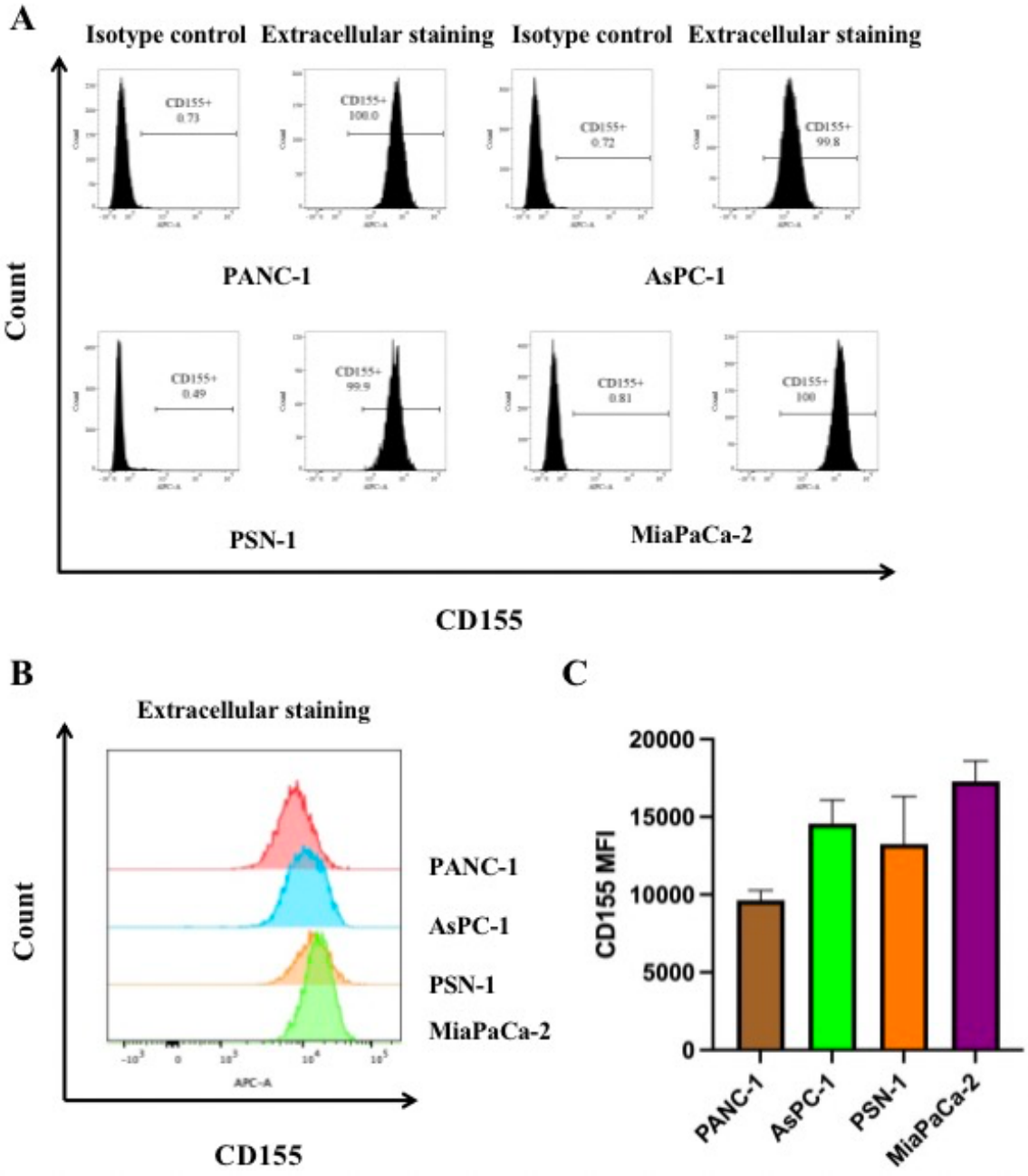

Figure 10: CD155 expression on pancreatic cancer cell lines was measured using FACS analysis

(A) Detection of CD155 fluorescence signal positive cells on four pancreatic cancer cell lines using FACS analysis. We used APC-conjugated anti-CD155 to identify extracellular expression of CD155; (B) The median fluorescence intensity (MFI) of CD155 fluorescence signal positive cells on four pancreatic cancer cell lines using FACS analysis; (C) Column graph of statistical analysis of the MFI of CD155 fluorescence signal positive cells on four pancreatic cancer cell lines using FACS analysis. Three independent repeated experiments were performed. All data are presented as mean ± SD.

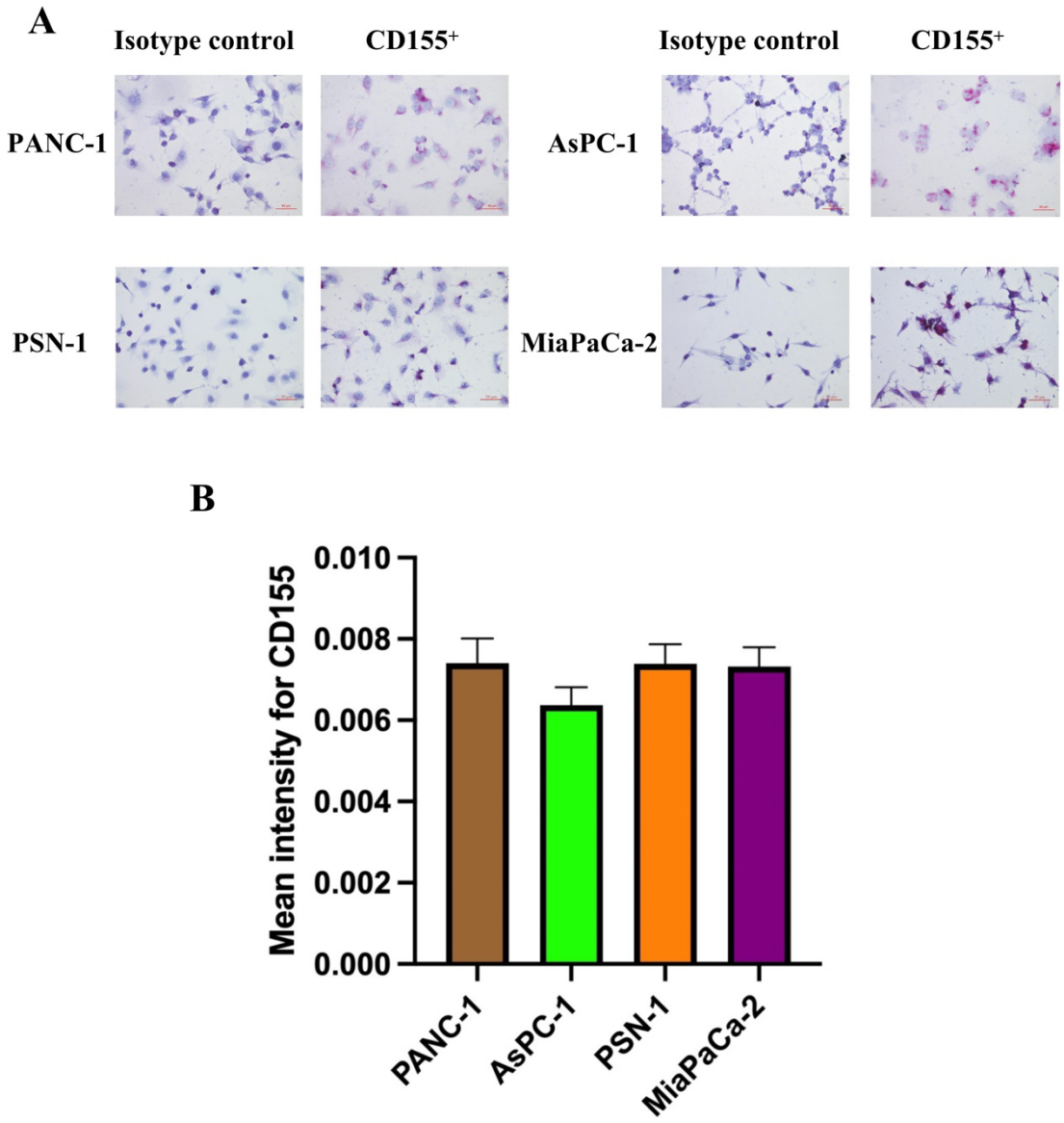

Figure 11: CD155 expression on pancreatic cancer cells was measured by ICC

(A) ICC representative pictures of CD155 staining signal positive cells of four pancreatic cancer cell lines under 20x magnification; (B) Column graph of CD155 staining signal positive cells of four pancreatic cancer cell lines by using Image-Pro Plus 6.0 to quantify them. Three independent repeated experiments were performed. All data are presented as mean ± SD.

CD155 expression	PANC-1	AsPC-1	PSN-1	MiaPaCa-2
qPCR analysis	+	+	+	+
FACS analysis	+	+	+	+
ICC	+	+	+	+

Table 10: Summary of CD155 expression on pancreatic cancer cells by qPCR analysis, FACS analysis and ICC

+ means CD155 expression can be detected.

3.3 Treatment of activated PBMCs with CD155 recombinant protein reduced the IFN-γ production in activated $CD8^+CD96^+$ T cells

For the $CD8^+$ T cell activation, the Leukocyte Activation Cocktail was used. Additional stimulation with CD155 recombinant protein was performed as described in Material and Methods. The gating strategy is as shown in *Figure 12*. First, $CD45^+$ cells were gated from PBMCs. Subsequently, $CD3^+CD96^+$ cells were gated from the $CD45^+$ population. From $CD3^+CD96^+$ population, $CD8^+$ cells were selected, and the expression level of IFN-γ in the $CD8^+CD96^+$ population was analyzed. After adding 5 µg/ml of CD155 recombinant protein to stimulated PBMCs, a reduction in IFN-γ expression was observed in activated $CD8^+$ T cells, indicating that when CD96 binds to its ligand CD155, the production of IFN-γ of $CD8^+$ T cells decreased. It is noteworthy that the higher concentrations of CD155 recombinant protein did not reduce IFN-γ production more (*Figure 13*).

Therefore, this experiment demonstrated that binding of CD96 to its ligand CD155 led to decreased IFN-γ expression in activated $CD8^+$ T cells.

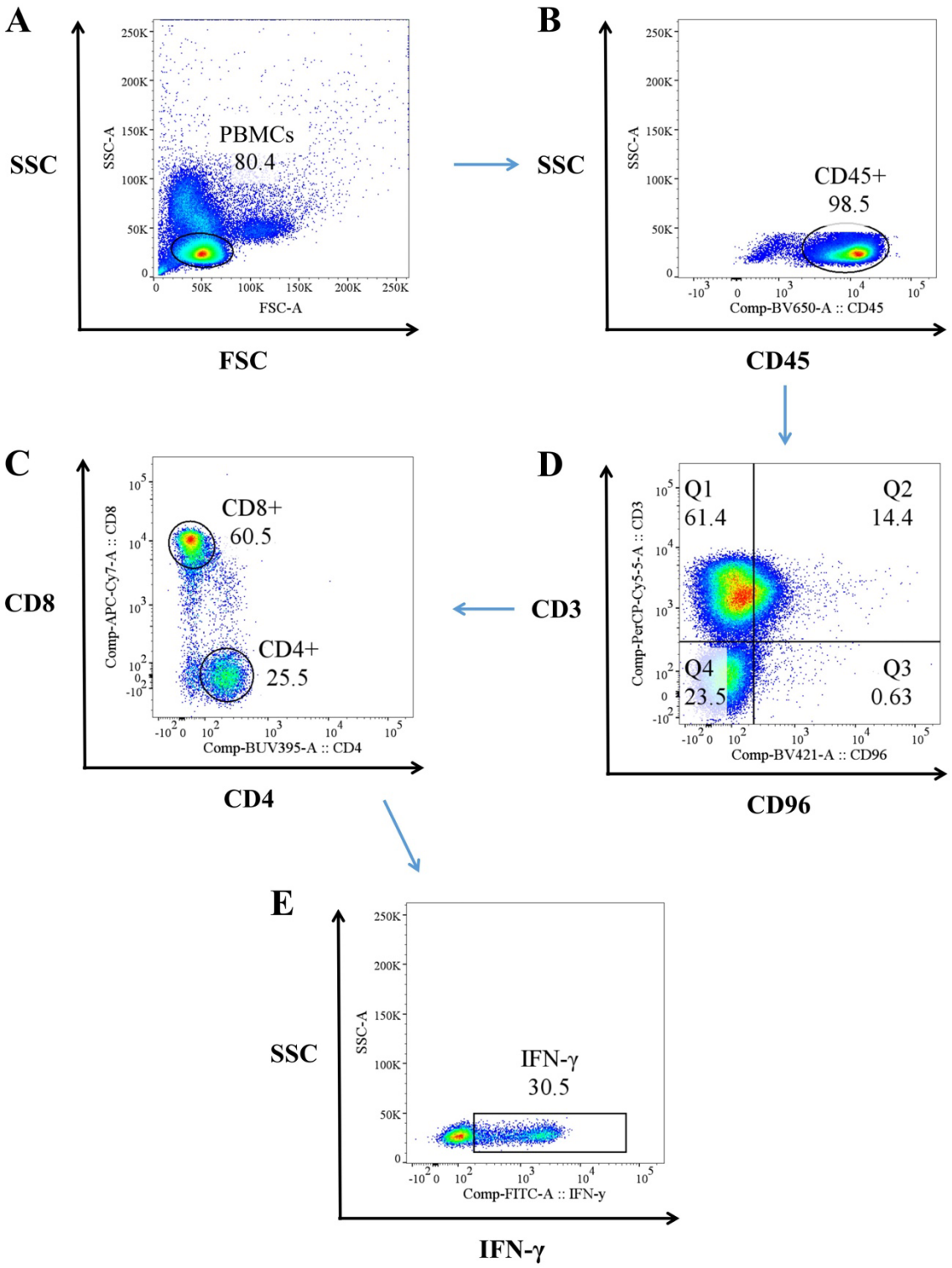

Figure 12: Gating strategy for detecting IFN-γ expression level in activated $CD8^+CD96^+$ T cells using FACS analysis

(A) PBMCs in gate; (B) $CD45^+$ cells in gate; (C) $CD8^+CD96^+$ and $CD4^+CD96^+$ T cells in gates from $CD3^+CD96^+$ T cells; (D) $CD3^+CD96^+$ T cells in Q2; (E) IFN-γ fluorescence signal positive cells of activated

$CD8^+CD96^+$ T cells in gate.

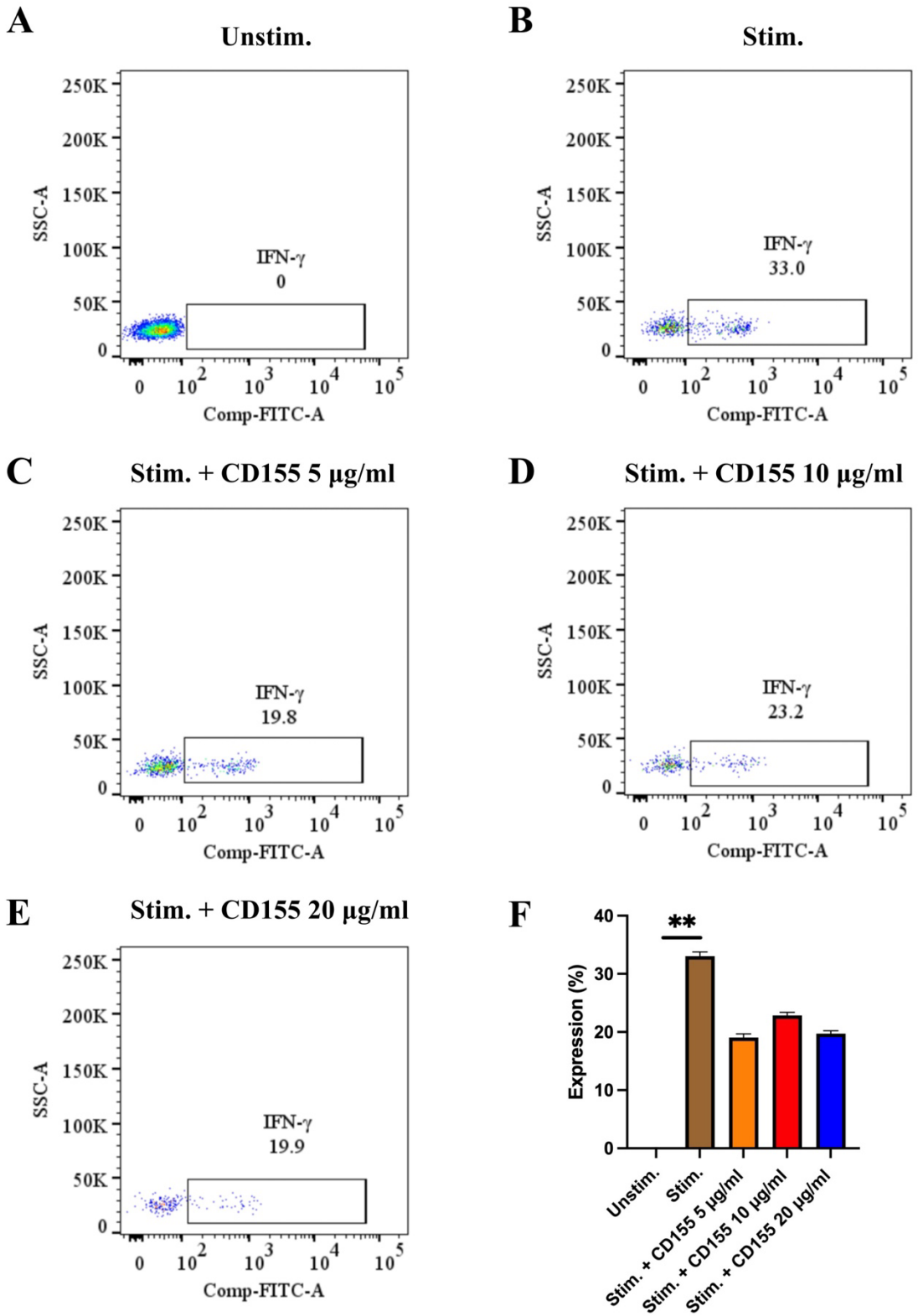

Figure 13: IFN-γ fluorescence signal detection in $CD8^+CD96^+$ T cells using FACS analysis

Detection of IFN-γ fluorescence signal positive cells of $CD8^+$ T cells in unstimulated PBMCs (A), activated PBMCs with 0 μg/ml (B), 5 μg/ml (C), 10 μg/ml (D), and 20 μg/ml (E) CD155 recombinant protein after culturing 4 hours; (F) Column graph of statistical analysis of IFN-γ fluorescence signal positive cells of $CD8^+$ T cells in five groups. Three independent repeated experiments were performed. All data are presented as mean ± SD; P-values were calculated by non-parametric Kruskal-Wallis followed by Dunn's test. (**=$P<0.01$).

Unstim. means Unstimulated PBMCs; Stim. means Stimulated PBMCs.

3.4 Blocking the interaction between CD96 and CD155 restored the IFN-γ production in activated $CD8^+CD96^+$ T cells

To determine whether IFN-γ production in activated $CD8^+$ T cells could be restored by blocking the connection between CD96 and CD155, CD155 recombinant protein and CD96 antibodies were added into activated PBMCs with stimulation. After 4 hours incubation, FACS analysis was performed to assess IFN-γ production in activated $CD8^+$ T cells. The gating strategy is as shown in Figure 11. First, $CD45^+$ cells were gated from PBMCs. Subsequently, $CD3^+CD96^+$ cells were gated from the $CD45^+$ population. From $CD3^+CD96^+$ population, $CD8^+$ T cells were selected, and the expression level of IFN-γ in the $CD8^+$ T cells was analyzed. The findings showed that, following the addition of CD96 antibodies, activated $CD8^+$ T cells expressed more IFN-γ compared to those without CD96 antibodies (*Figure 14*).

In conclusion, our experiments demonstrated that blocking the interaction between CD96 and CD155 can effectively restore IFN-γ production in activated $CD8^+$ T cells.

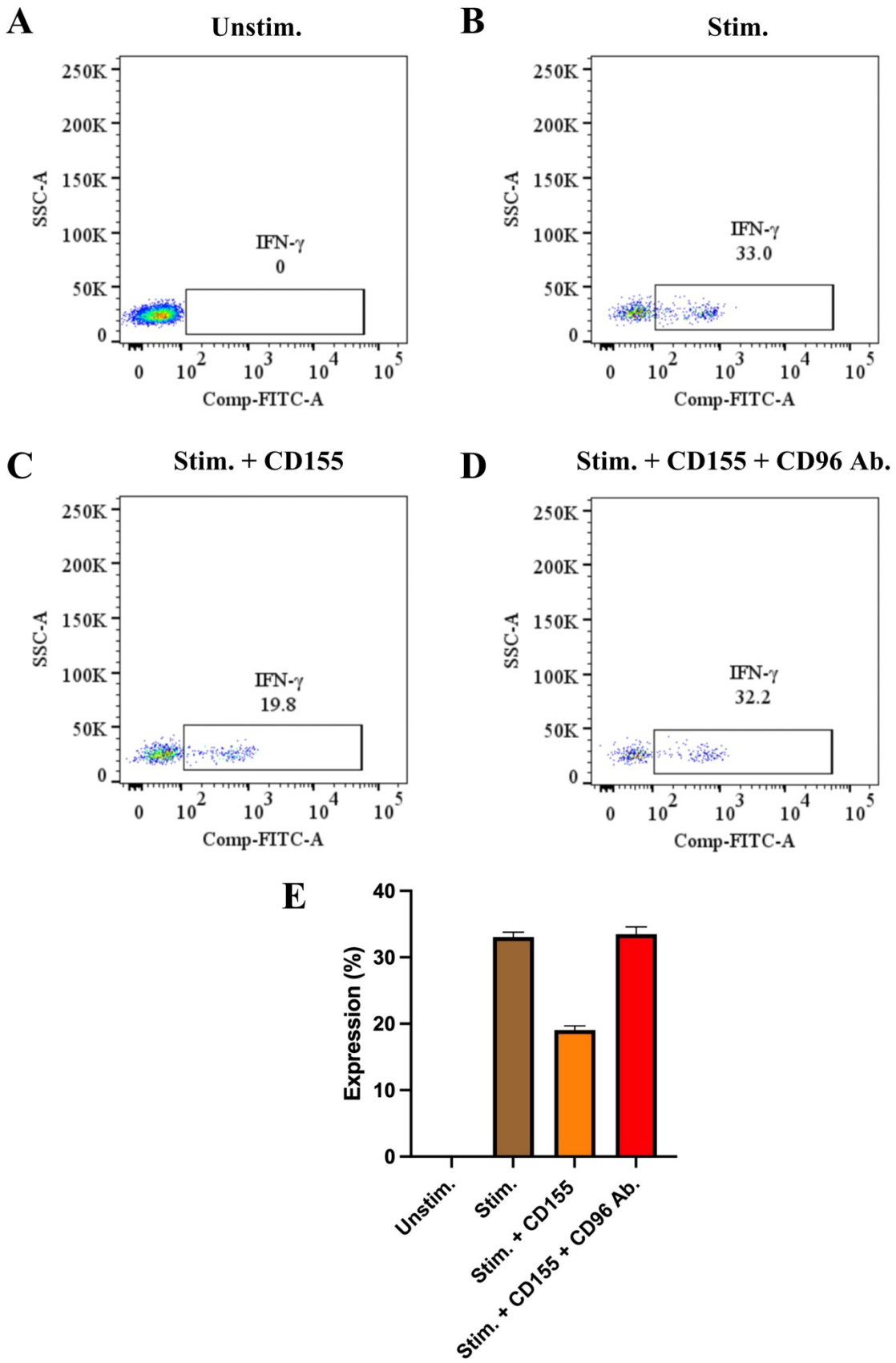
A
Unstim.
B
Stim.
C
Stim. + CD155
D
Stim. + CD155 + CD96 Ab.
SSC-A
Comp-FITC-A
IFN-γ
0
IFN-γ
33.0
IFN-γ
19.8
IFN-γ
32.2
250K
200K
150K
100K
50K
0
10^2
10^3
10^4
10^5
E
Expression (%)
40
30
20
10
0
Unstim.
Stim.
Stim. + CD155
Stim. + CD155 + CD96 Ab.

Figure 14: FACS analysis of IFN-γ expression levels in $CD8^+CD96^+$ T cells

Detection of IFN-γ fluorescence signal positive cells of $CD8^+$ T cells in unstimulated PBMCs (A), activated PBMCs with 0 μg/ml CD155 recombinant protein (B), activated PBMC with 5 μg/ml CD155 recombinant protein (C), and activated PBMCs with 5 μg/ml CD155 recombinant protein as well as 5 μg/ml CD96 antibodies (D); (E) Column graph of IFN-γ fluorescence signal positive cells in $CD8^+$ T cells among four groups. Three independent repeated experiments were performed. All data are presented as mean ± SD.

Unstim. means Unstimulated PBMCs; Stim. means Stimulated PBMCs; Ab. means Antibodies.

3.5 Blocking the binding of CD96 and CD155 restored the mitochondrial respiratory activity of activated PBMCs

As mentioned in the introduction part, immune checkpoints can affect the killing ability of immune cells by affecting the respiratory metabolism of immune cells, this experiment aimed to investigate changes in mitochondrial respiration function after adding CD155 recombinant protein and CD96 antibodies to stimulated PBMCs. The Seahorse XFp analyzer was used to measure mitochondrial oxygen consumption rate (OCR) using Seahorse XFp Real-Time ATP Rate Assay Kit as described in Material and Methods. The results demonstrated a significant increase in OCR in stimulated PBMCs compared to unstimulated PBMCs. However, following the addition of CD155 recombinant protein, the OCR of PBMCs decreased. However, the subsequent addition of CD96 antibodies restored the OCR of stimulated PBMCs to a relatively high level, indicating that blocking the interaction between CD96 and CD155 can reverse the inhibitory effect on mitochondrial respiration (*Figure 15*).

These findings suggested that anti-CD96 antibodies could enhance the

metabolic activity of stimulated PBMCs by disrupting the interaction between CD96 and CD155.

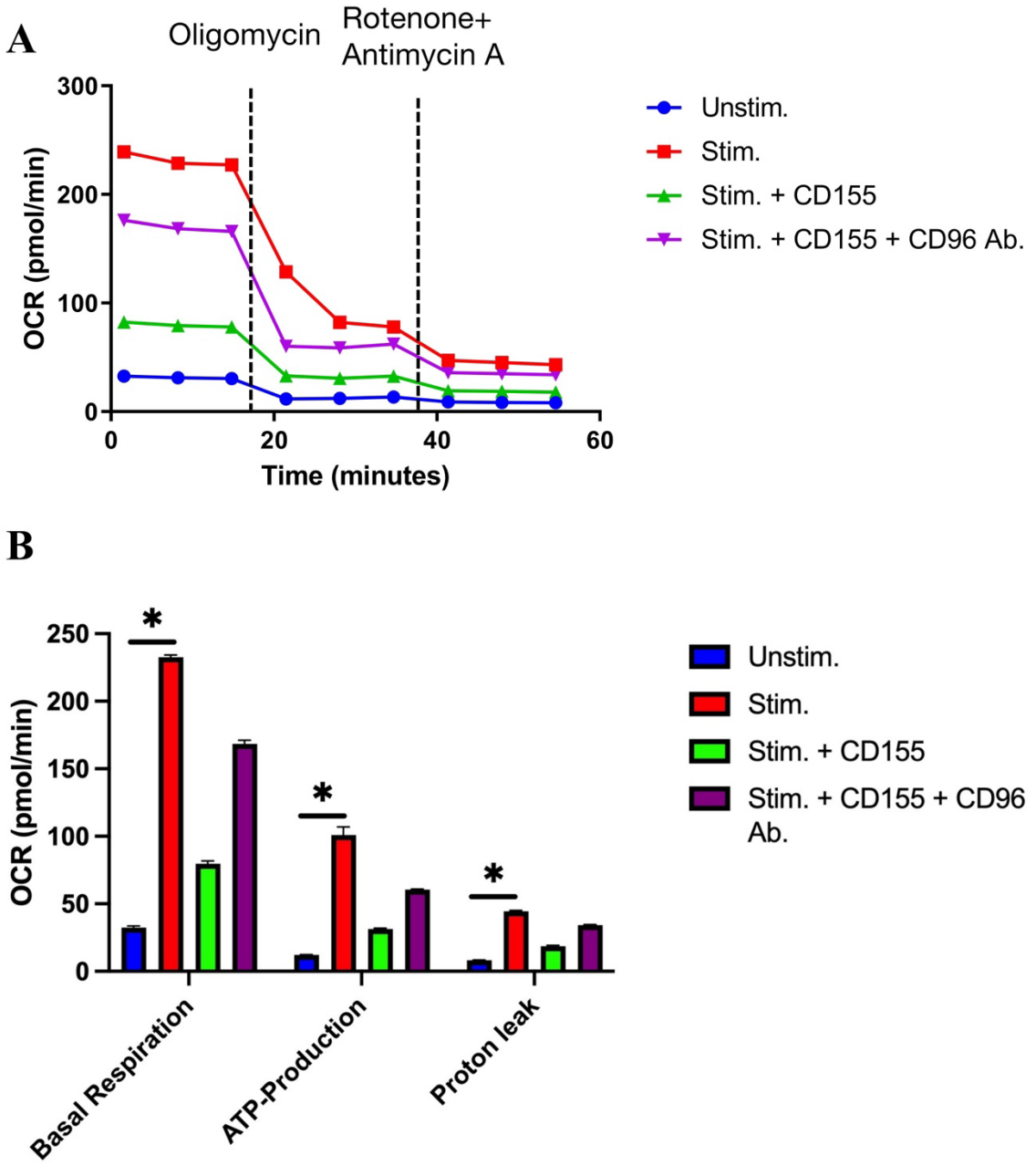

Figure 15: Mitochondrial metabolism analysis of PBMCs

(A) OCR values obtained during mitochondrial metabolism test measured by Seahorse XFp Analyzer in unstimulated PBMCs, stimulated PBMCs, stimulated PBMCs cultured with 5 µg/ml CD155 recombinant protein, stimulated PBMCs cultured with 5 µg/ml CD155 recombinant protein as well as 5 µg/ml CD96 antibodies, performed by injection of oligomycin and rotenone + antimycin A (Rot/AA); (B) Column graph of statistical analysis

of OCR values from four different treatment groups. Three independent repeated experiments were performed. All data are presented as mean ± SD; P-values were calculated by non-parametric Kruskal-Wallis followed by Dunn's test. (*=P<0.05).

Unstim. means Unstimulated PBMCs; Stim. means Stimulated PBMCs; Ab. means Antibodies.

3.6 Bioinformatic analysis demonstrated higher production of CD96 and CD155 in pancreatic cancer tissues compared to healthy pancreas tissues

To study the expression level of CD96 and CD155 in pancreatic cancer tissues, the bioinformatic analysis online platform GEPIA (http://gepia.cancer-pku.cn/index.html) was utilized to assess the CD96 and CD155 production level in pancreatic cancer using the TCGA database. The findings demonstrated that the level of expression of CD96 as well as CD155 was higher in pancreatic cancer tissues compared to normal pancreatic tissues (*Figure 16 A-D*). The significant overexpression of CD96 and CD155 in pancreatic cancer suggests CD96 is potential to be a possible immune checkpoint inhibitor. The overall survival of pancreatic cancer patients with different levels of CD96 expression was then evaluated, the findings indicated that between the 40th and 70th months after diagnosis, patients with low CD96 expression had a higher survival rate compared to those with high CD96 expression. (*Figure 16 E*).

Overall, the results of bioinformatic analysis show that a higher production of CD96 and CD155 in pancreatic cancer tissues than in normal pancreatic tissues.

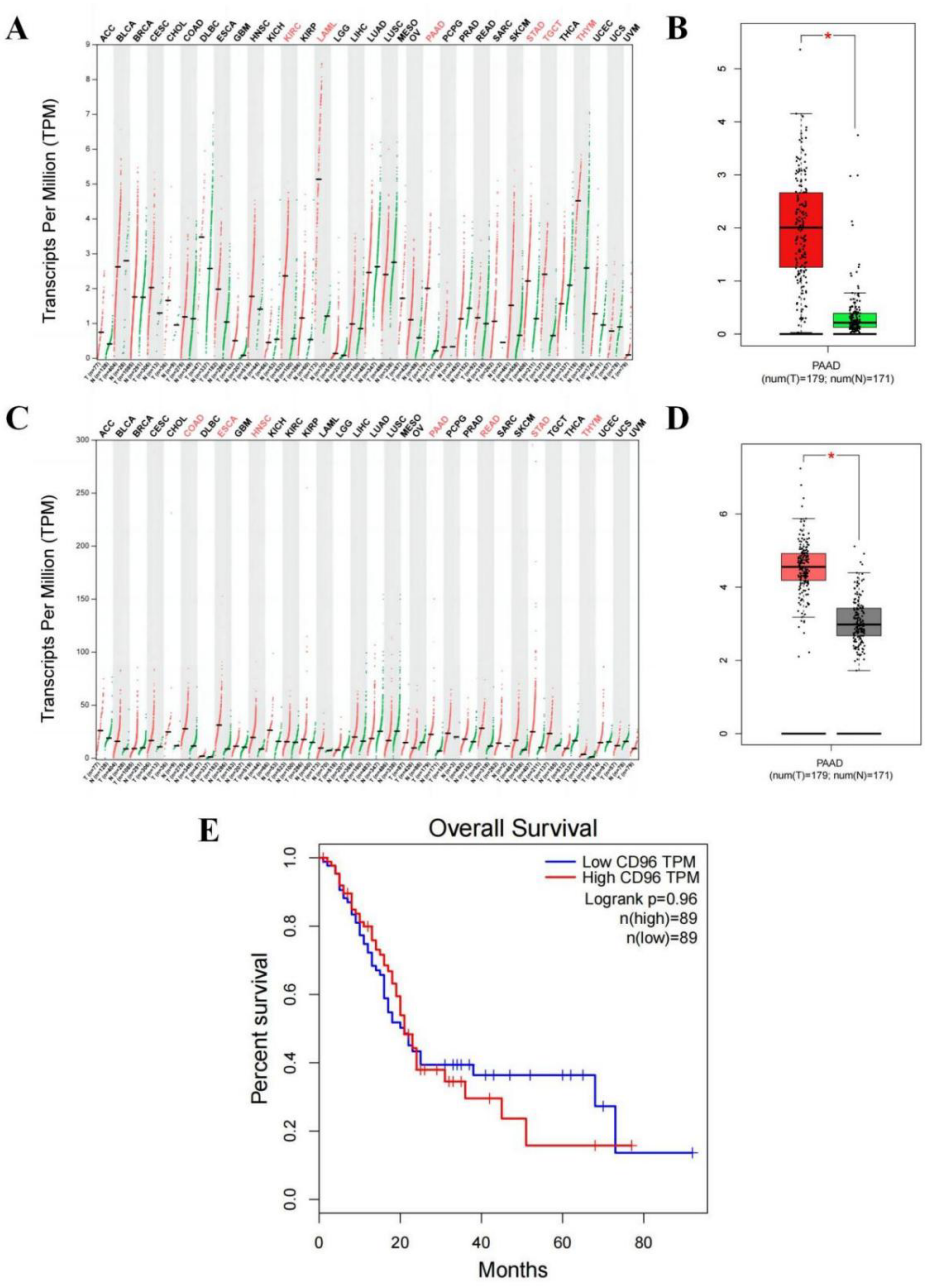

Figure 16: Bioinformatic analysis of CD96 and CD155

(A) CD96 expression among the pan-cancer. (B) Box plot of CD96 expression in pancreatic cancer tissues (red bar) and normal pancreatic tissues (green bar). (C) CD155 expression among the pan-cancer. (D) Box plot of CD155 expression in pancreatic cancer tissues (red bar) and normal pancreatic tissues (gray bar). (E) The overall survival in pancreatic cancer patients with low and high CD96 expression. P-values were calculated by Mann-Whitney test. (*=P<0.05).

PAAD means pancreatic adenocarcinoma; num(T) means number of

pancreatic cancer tissues; num(N) means number of normal pancreatic tissues; n(high) means number of pancreatic cancer patients' tissue with high CD96 expression; n(low) means number of pancreatic cancer patients' tissue with low CD96 expression.

4. Discussion

4.1 Current research and gaps in the study of CD96

Immune checkpoint blockers are promising medications to change the cancer treatments' methods [107]. These medications were developed based on an in-depth knowledge of how the body's immune response works and how tumors avoid immune system's attack. Immune checkpoint blockers are designed to improve the ability of the immune system to kill cancer cells by removing the immune system's inhibition caused by the connection between the immune checkpoints and their ligands.

Current immune checkpoint inhibitor drugs mainly target the CTLA-4 and PD-1. Because cancers are so diverse and complicated, existing immune checkpoint inhibitors sometimes do not achieve optimal efficacy. Consequently, the discovery of novel immune checkpoint blockers is critical. CD96, as a potential immune checkpoint blocker, has already attracted the attention of some scientists and is beginning to be studied [57].

Most studies about the impact of CD96 on cancers were conducted in cell and animal experiments. Animal experiments demonstrated that combination inhibition of PD-1 and CD96 greatly increased the immunological effectiveness of $CD8^+$ T cells compared with PD-1 blockage alone, thereby inhibiting the growth of cervical cancer [69]. Research by Mark J. Smyth et al. suggested that combing CD96 inhibitor with PD-1 or CTLA-4 inhibitor could increase immune cells' anti-cancer capacity in melanoma, colorectal cancer and fibrosarcoma [100]. Another study shown that inhibiting the connection between CD96 and CD155 could kill liver cancer cells by restoring the immune activity of NK cells [70]. Moreover, CD96 has anti-inflammatory properties and can facilitate the attachment of NK cells to other cells expressed CD155. This enhances the exchange of surface chemicals between NK cells and other cells and improves the toxic capacity of activated NK cells [96, 99,108].

In summary, existing research has confirmed the possible role of CD96 as an

immune checkpoint blocker in various cancers. However, there are no further basic studies on CD96 and pancreatic cancer, so we started our research.

4.2 Regulation network of CD96 and CD155

In order to study CD96 in depth, it is necessary to have a certain understanding of the regulatory network in which CD96 resides. The main receptor of CD96 is CD155, and the receptor of TIGIT and CD226 is also CD155, they form this regulatory network together[58]. TIGIT, found in 2009, relates to the immunoglobulin family [109], which is a kind of co-stimulatory immune checkpoint, expressed mainly in lymphocytes such as $CD8^+$ T cells and NK cells [110]. TIGIT's receptors include CD155, CD112, as well as CD113, while TIGIT having the highest selective affinity to CD155 among them. Therefore, we primarily focus on the regulatory relationship between TIGIT and CD155 [109]. CD226 is a kind of co-stimulatory checkpoint, which relates to the immunoglobulin family [111]. CD226 enhances immune cell efficiency by promoting adhesion between immune cells and other cells [112]. Furthermore, CD226 can promote the production of toxic substances, including as perforin and granzymes, by NK cells, leading to death of target cells [113]. CD226 can also interact with antigen-presenting cells to induce T cell activation [114]. In summary, CD226 can promote immune cell’s activation and cytotoxicity through multiple signaling pathways.

The selective affinities in TIGIT and CD155, CD96 and CD155, CD226 and CD155 are 1-3 nmol/L, 37.6 nmol/L, and 114-199 nmol/L, respectively. This indicates that TIGIT has the strongest selective affinity, while CD96 has moderate selective affinity, and CD226 has the weakest selective affinity [57]. In the normal situation, the interaction of CD96, TIGIT, CD226, and CD155 ensures moderate activation of the immune system, preventing an excessive immune response while maintaining effective resistance against infections and tumor cells. In the case of CD155 overexpression, such as the rapid growth of CD155-expressing tumors, this balance is be broken due to the high affinity of TIGIT and CD96 to CD155, and the immune cells’

function is inhibited, allowing the tumor to escape immune surveillance [57].

4.3 Further discussion of our results

We detected CD96 expression on $CD8^+$ T cells using FACS analysis, then discovered that CD96 expression in unstimulated $CD8^+$ T cells was higher than that in stimulated $CD8^+$ T cells. These results are consistent with earlier research [67]. One study found that CD96 expression was also downregulated in stimulated NK cells compared with unstimulated NK cells [115]. There is no research on why CD96 expression is downregulated in stimulated immune cells compared with unstimulated immune cells. At the same time, some studies have shown that the production of other immune checkpoints, like PD-1, is also reduced in stimulated $CD8^+$ T cells compared with unstimulated $CD8^+$ T cells, the mechanism is that IL12 secreted by antigen presenting cells decreases the PD-1's production in stimulated $CD8^+$ T cells [116].

We observed the production of CD155 among four pancreatic cancer cell lines using qPCR, ICC, and FACS analysis. The CD155 mRNA expression can be detected in the pancreatic cancer cell lines PANC-1 and MiaPaCa-2 using qPCR analysis were consistent with previous findings [81] (Table 11), we conducted the FACS analysis and ICC about it because the location of CD155 protein can be only detected by FACS analysis and ICC. To our knowledge, this is the first study to report CD155 expression in the AsPC-1 and PSN-1 pancreatic cancer cell lines. Given the production of CD155 in pancreatic cancer cells, our results provide the potential basis for further investigation about CD96 can be an immune checkpoint inhibitor for pancreatic cancer treatment. Finally, we reviewed the literature and found that CD155 can also be expressed in other cancer cells, like lung and colorectal cancer, indicating the potential of inhibiting the CD96/CD155 axis in other cancers [117-119].

	PANC-1	AsPC-1	PSN-1	MiaPaCa-2
qPCR analysis	√	-	-	√
FACS analysis	-	-	-	-
ICC	-	-	-	-

Table 11: Summary of published data of CD155 expression on pancreatic cancer cells

√ means CD155 expression can be detected on this pancreatic cancer cell line by this method has been published; - means CD155 expression can be detected on the pancreatic cancer cell line by this method has not been published.

Besides, adding CD155 recombinant protein to stimulated PBMCs could reduce the immune efficacy of stimulated $CD8^+$ T cells. However, this immune efficacy could be restored by blocking CD96. We didn't find any studies regarding this result until now. It is worth noting that current studies have investigated the blockade of CD96 to improve $CD8^+$ T cell immune efficacy in colorectal cancer, melanoma, fibrosarcoma, and cervical cancer, but no research has been conducted on pancreatic cancer. These studies' results showed the immune efficacy of inhibiting CD96 consistent with our research. It is worth mentioning that their research has already been carried out in animal experiments, while ours was still at the level of cell experiments, therefore, further animal experiments are necessary to investigate the efficacy of CD96 inhibition among pancreatic cancer [69, 100]. Another noteworthy point is that when we cultured stimulated PBMCs with different concentrations of CD155 recombinant protein, we found that stimulated $CD8^+$ T cells produced lower IFN-γ than those without CD155 recombinant protein, however, higher concentrations of CD155 recombinant

protein did not reduce IFN-γ expression more, the possible reason is 5μg/ml CD155 recombinant protein was sufficient to combine with its ligand.

We found that, in vitro, the mitochondrial respiratory function of PBMCs significantly decreased after the addition of CD155 recombinant protein. However, this respiratory function recovered after CD96 antibodies were added. This result demonstrated that inhibiting the connection between CD96 and CD155 could restore metabolic capacity of PBMCs, thereby enhancing their immune function. So far, there are no studies to show this. Similar study was conducted on PD-1, and the decrease in the respiration capacity of $CD8^+$ T cells was observed after the addition of PD-L1, the ligand of PD-1, but no PD-1 or PD-L1 blockers were included in this study to see whether the metabolic capacity of $CD8^+$ T cells would recover [120].

Bioinformatic analysis showed that CD155 is expressed at a higher level in pancreatic cancer tissues than adjacent non-cancerous tissues. This result is consistent with other studies [81, 121]. At the same time, studies shown that CD155 is also abundantly produced in different cancer tissues, like breast and colorectal cancer tissues [122, 123]. Additionally, CD96 expression is also higher in pancreatic cancer tissues than adjacent tissues, aligning with existing research results [72]. Studies shown that CD96 is also abundantly produced in other cancer tissues, like liver cancer, cervical cancer, and glioma [72, 124]. Previous research found that the immune checkpoint and its ligand (TIGIT and CD155) were abundantly produced in pancreatic cancer tissues and could boost immune evasion in pancreatic cancer. Inhibiting the connection between TIGIT and CD155 can produce an anti-cancer effect, which indicated the feasibility of targeting CD96 in immunotherapy for pancreatic cancer due to the high expression of CD96 and CD155 in pancreatic cancer tissues [125-126]. Although current research found no significant link between CD96 expression and pancreatic cancer prognosis, patients who have a higher CD96 level exhibit a poorer prognosis between 40th and 70th months after being diagnosed. This suggests the possibility of treating anti-CD96 therapy to pancreatic cancer patients during

this period. Some research pointed out that high production of CD96 related to the bad prognosis of liver cancer and gastric cancer, so that high production of CD96 often predicted the bad outcome of cancer patients [70, 127]. However, one study showed that CD96 expression was linked to the good prognosis of pancreatic cancer [128], the reason for the inconsistency may be this study only concentrated on early stage of pancreatic cancer tissues, but the production of molecule at early and late stages of pancreatic cancer tissues might be different. The formation of cancer tissues is an ongoing procedure that involves changes in many genes and molecular pathways, which can result in large expression variations of the molecule at different stages [129].

In conclusion, CD96 can be regarded as a potential immunotherapeutic target for treating pancreatic cancer through our work.

4.4 Limitations of the study

During the research process, some limitations were encountered. The experiments were conducted in the cellular level, although blocking CD96 in cell experiments did restore immune cells' function, we aspire to validate these findings in mouse models in the future research, because animal experiments, compared to cell experiments, can better simulate the complexity of living organisms, including organ systems, cellular interactions and immune responses, and animal models have a better physiological and anatomical structures to enhance the accuracy of predicting biological effects in humans. This will provide greater support for the potential clinical application of our research results [130-132].

Regarding the source of CD155 which was used to connect to CD96, we used CD155 recombinant protein which is a straightforward and reliable method. While this is the simplest way to facilitate the connection between CD96 and CD155, it does not accurately imitate the interactions among immune cells and tumor cells in the human body. Co-culturing pancreatic cancer cell lines with immune cells might be a more effective approach [133]. Tumor organoid is another promising method [134]. Tumor organoid is three-

dimensional tissue structures cultured in vitro, derived from patient tumor tissues or tumor stem cells. The organoid can better simulate the complex cellular composition and microenvironment of original tumors [135]. Using CD96 inhibitors in conjunction with tumor organoid might better simulate the effects of CD96 blockade in the human body.

The primary ligand for CD96 is CD155 which expressed in various cancer cells [136]. Through bioinformatic analysis of TCGA datasets, we observed high expression level of CD155 within cancer tissues compared with their corresponding normal tissues, including pancreas, colorectum, kidney, esophagus, head and neck, and stomach [137]. And high expression level of CD96 was observed in cancer tissues compared with their corresponding normal tissues, including pancreas, kidney, breast, cervix, colorectum, esophagus, and stomach [72]. It suggested that blocking CD96 might have potential applications in pancreatic cancer, colorectal cancer, kidney cancer, esophageal cancer, and gastric cancer. Our investigations only confirmed CD155 expression in four pancreatic cancer cell lines, but not in colorectal cancer, kidney cancer, esophageal cancer, and gastric cancer cell lines. This is a limitation of our experiment, and further research is needed to solve this deficiency.

CD96 is a relatively new and less studied immune checkpoint compared to well-established ones like PD-1 and CTLA-4. There is much research work to be done, and its effectiveness in human body remains uncertain. Currently, we hope CD96 can be served as an adjuvant drug to other drugs. Existing research indicates that combination treatment frequently produces more successful immune effects than single drug therapy [138]. While we observed that the effectiveness in cell experiments when using CD96 antibody alone in pancreatic cancer cell lines, the impact of combination treatment between CD96 antibody and additional treatment medicines, like PD-1 inhibitor or chemotherapeutic medicine, is uncertain. We aim to further explore its role in future research.

4.5 Prospects for CD96 as a potential immunotherapy target

The immunotherapy for cancer is a novel method to treat cancer which utilizes the individual's natural immunity to defend the illness [139]. Anti-PD-1 or anti-PD-L1 antibodies are currently the most successful immunotherapeutic drugs [140]. They work by inhibiting immunological checkpoint routes, restoring immune cells' cytotoxicity against cancer cells. However, the effectiveness of taking only one immune checkpoint inhibitor is restricted, and combination therapy often yields better results [141]. CD96 is an immune checkpoint that still offers considerable research potential. Some studies suggested that combining PD-1 inhibitors with CD96 inhibitors could be used to treat cervical cancer [69]. The use of CD96 inhibitors can enhance the chemotherapeutic effects in patient-derived breast tumor transplantation models [142] and blocking CD96 can reverse immune suppression and poor prognosis in lung adenocarcinoma [68]. Bioinformatic analysis also suggested that CD96 activity could stimulate immune invasion and impact the prognosis of some cancer patients, demonstrating CD96 can be regarded as a potential predictive marker [72].

To summarize, research on CD96 remains in its early stages when compared to more developed immune checkpoint blockers like PD-1. Most studies were limited to several types of cancers, focusing on basic cellular and animal experiments. There is a lack of foundational experimental validation across a broader range of cancers and sufficient evidence from basic clinical trials. We are still in need of conclusive proof to demonstrate the effectiveness of CD96 inhibitor in cancer patients and their combination treatment with other treatment medicines. Although there is significant space for further exploration in CD96 research, the observed data indicate that CD96 possesses potential as a new immunotherapeutic agent.

5. Conclusion

In conclusion, CD96 could be recognized as a potential immunotherapy target for pancreatic cancer. Through immunotherapeutic strategies of targeting CD96, there is optimism for enhancing the outlook and survival rates of pancreatic cancer patients. However, further research and validation, including preclinical studies and clinical trials, are still necessary for clinical application. A comprehensive grasp of the immune regulatory mechanisms of CD96 and the optimization of treatment plans may provide a better method to the immunotherapy of pancreatic cancer.

6. References

1. Hadden M, Mittal A, Samra J, Zreiqat H, Sahni S, Ramaswamy Y. Mechanically stressed cancer microenvironment: Role in pancreatic cancer progression. Biochim Biophys Acta Rev Cancer. 2020 Dec;1874(2):188418.

2. Vincent A, Herman J, Schulick R, Hruban RH, Goggins M. Pancreatic cancer. Lancet. 2011 Aug 13;378(9791):607-20.

3. Rawla P, Sunkara T, Gaduputi V. Epidemiology of Pancreatic Cancer: Global Trends, Etiology and Risk Factors. World J Oncol. 2019 Feb;10(1):10-27.

4. Yuan C, Kim J, Wang QL, Lee AA, Babic A; PanScan/PanC4 I-III Consortium; Amundadottir LT, Klein AP, Li D, McCullough ML, Petersen GM, Risch HA, Stolzenberg-Solomon RZ, Perez K, Ng K, Giovannucci EL, Stampfer MJ, Kraft P, Wolpin BM. The age-dependent association of risk factors with pancreatic cancer. Ann Oncol. 2022 Jul;33(7):693-701.

5. Lee CJ, Scheiman J, Anderson MA, Hines OJ, Reber HA, Farrell J, Kochman ML, Foley PJ, Drebin J, Oh YS, Ginsberg G, Ahmad N, Merchant NB, Isbell J, Parikh AA, Stokes JB, Bauer T, Adams RB, Simeone DM. Risk of malignancy in resected cystic tumors of the pancreas < or =3 cm in size: is it safe to observe asymptomatic patients? A multi-institutional report. J Gastrointest Surg. 2008 Feb;12(2):234-42.

6. Hjaltelin JX, Novitski SI, Jørgensen IF, Siggaard T, Vulpius SA, Westergaard D, Johansen JS, Chen IM, Juhl Jensen L, Brunak S. Pancreatic cancer symptom trajectories from Danish registry data and free text in electronic health records. Elife. 2023 Nov 21;12:e84919.

7. Parbhu SK, Adler DG. Pancreatic neuroendocrine tumors: contemporary diagnosis and management. Hosp Pract (1995). 2016 Aug;44(3):109-19.

8. Hu JX, Zhao CF, Chen WB, Liu QC, Li QW, Lin YY, Gao F. Pancreatic cancer: A review of epidemiology, trend, and risk factors. World J Gastroenterol. 2021 Jul 21;27(27):4298-4321.

9. Ilic M, Ilic I. Epidemiology of pancreatic cancer. World J Gastroenterol. 2016 Nov 28;22(44):9694-9705.

10. Li J, Li Y, Chen C, Guo J, Qiao M, Lyu J. Recent estimates and predictions of 5-year survival rate in patients with pancreatic cancer: A model-based period analysis. Front Med (Lausanne). 2022 Dec 8;9:1049136.

11. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. CA Cancer J Clin. 2020

Jan;70(1):7-30.

12. Jemal A, Siegel R, Ward E, Murray T, Xu J, Smigal C, Thun MJ. Cancer statistics, 2006. CA Cancer J Clin. 2006 Mar-Apr;56(2):106-30.

13. McGuigan A, Kelly P, Turkington RC, Jones C, Coleman HG, McCain RS. Pancreatic cancer: A review of clinical diagnosis, epidemiology, treatment and outcomes. World J Gastroenterol. 2018 Nov 21;24(43):4846-4861.

14. Lohiya A, Daniel RA, Smith RD, Nagar M, Shankar A, Lahariya C. Cancer prevention and control in India can get a boost through primary health care-based approach: A review. J Family Med Prim Care. 2022 Aug;11(8):4286-4292.

15. Sasajima J, Okamoto K, Taniguchi M. Hematogenous Gastric Metastasis of Pancreatic Cancer. Case Rep Gastroenterol. 2016 May 19;10(1):75-80.

16. Singhi AD, Koay EJ, Chari ST, Maitra A. Early Detection of Pancreatic Cancer: Opportunities and Challenges. Gastroenterology. 2019 May;156(7):2024-2040.

17. Bures J, Kohoutova D, Skrha J, Bunganic B, Ngo O, Suchanek S, Skrha P, Zavoral M. Diabetes Mellitus in Pancreatic Cancer: A Distinct Approach to Older Subjects with New-Onset Diabetes Mellitus. Cancers (Basel). 2023 Jul 19;15(14):3669.

18. Overbeek KA, Levink IJM, Koopmann BDM, Harinck F, Konings ICAW, Ausems MGEM, Wagner A, Fockens P, van Eijck CH, Groot Koerkamp B, Busch ORC, Besselink MG, Bastiaansen BAJ, van Driel LMJW, Erler NS, Vleggaar FP, Poley JW, Cahen DL, van Hooft JE, Bruno MJ; Dutch Familial Pancreatic Cancer Surveillance Study Group. Long-term yield of pancreatic cancer surveillance in high-risk individuals. Gut. 2022 Jun;71(6):1152-1160.

19. Carrato A, Falcone A, Ducreux M, Valle JW, Parnaby A, Djazouli K, Alnwick-Allu K, Hutchings A, Palaska C, Parthenaki I. A Systematic Review of the Burden of Pancreatic Cancer in Europe: Real-World Impact on Survival, Quality of Life and Costs. J Gastrointest Cancer. 2015 Sep;46(3):201-11.

20. Kardosh A, Lichtensztajn DY, Gubens MA, Kunz PL, Fisher GA, Clarke CA. Long-Term Survivors of Pancreatic Cancer: A California Population-Based Study. Pancreas. 2018 Sep;47(8):958-966.

21. Torphy RJ, Fujiwara Y, Schulick RD. Pancreatic cancer treatment: better, but a long way to go. Surg Today. 2020 Oct;50(10):1117-1125.

22. Bachmann J, Michalski CW, Martignoni ME, Büchler MW, Friess H. Pancreatic resection for pancreatic cancer. HPB (Oxford). 2006;8(5):346-51.

23. Wei K, Hackert T. Surgical Treatment of Pancreatic Ductal Adenocarcinoma. Cancers (Basel). 2021 Apr 20;13(8):1971.

24. Strobel O, Neoptolemos J, Jäger D, Büchler MW. Optimizing the outcomes of pancreatic cancer surgery. Nat Rev Clin Oncol. 2019 Jan;16(1):11-26.

25. Principe DR, Underwood PW, Korc M, Trevino JG, Munshi HG, Rana A. The Current Treatment Paradigm for Pancreatic Ductal Adenocarcinoma and Barriers to Therapeutic Efficacy. Front Oncol. 2021 Jul 15;11:688377.

26. Anand U, Dey A, Chandel AKS, Sanyal R, Mishra A, Pandey DK, De Falco V, Upadhyay A, Kandimalla R, Chaudhary A, Dhanjal JK, Dewanjee S, Vallamkondu J, Pérez de la Lastra JM. Cancer chemotherapy and beyond: Current status, drug candidates, associated risks and progress in targeted therapeutics. Genes Dis. 2022 Mar 18;10(4):1367-1401. doi: 10.1016/j.gendis.2022.02.007. Erratum in: Genes Dis. 2024 Jan 20;11(4):101211.

27. Jiang S, Fagman JB, Ma Y, Liu J, Vihav C, Engstrom C, Liu B, Chen C. A comprehensive review of pancreatic cancer and its therapeutic challenges. Aging (Albany NY). 2022 Sep 28;14(18):7635-7649.

28. Burris HA 3rd, Moore MJ, Andersen J, Green MR, Rothenberg ML, Modiano MR, Cripps MC, Portenoy RK, Storniolo AM, Tarassoff P, Nelson R, Dorr FA, Stephens CD, Von Hoff DD. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. J Clin Oncol. 1997 Jun;15(6):2403-13.

29. Neesse A, Michl P, Frese KK, Feig C, Cook N, Jacobetz MA, Lolkema MP, Buchholz M, Olive KP, Gress TM, Tuveson DA. Stromal biology and therapy in pancreatic cancer. Gut. 2011 Jun;60(6):861-8.

30. Von Hoff DD, Ervin T, Arena FP, Chiorean EG, Infante J, Moore M, Seay T, Tjulandin SA, Ma WW, Saleh MN, Harris M, Reni M, Dowden S, Laheru D, Bahary N, Ramanathan RK, Tabernero J, Hidalgo M, Goldstein D, Van Cutsem E, Wei X, Iglesias J, Renschler MF. Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. N Engl J Med. 2013 Oct 31;369(18):1691-703.

31. Oettle H, Riess H. Gemcitabine in combination with 5-fluorouracil with or without folinic acid in the treatment of pancreatic cancer. Cancer. 2002 Aug 15;95(4 Suppl):912-22.

32. Conroy T, Desseigne F, Ychou M, Bouché O, Guimbaud R, Bécouarn Y, Adenis A, Raoul JL, Gourgou-Bourgade S, de la Fouchardière C, Bennouna J, Bachet JB, Khemissa-

Akouz F, Péré-Vergé D, Delbaldo C, Assenat E, Chauffert B, Michel P, Montoto-Grillot C, Ducreux M; Groupe Tumeurs Digestives of Unicancer; PRODIGE Intergroup. FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. N Engl J Med. 2011 May 12;364(19):1817-25.

33. Brouwer TP, Vahrmeijer AL, de Miranda NFCC. Immunotherapy for pancreatic cancer: chasing the light at the end of the tunnel. Cell Oncol (Dordr). 2021 Apr;44(2):261-278.

34. Morrison AH, Byrne KT, Vonderheide RH. Immunotherapy and Prevention of Pancreatic Cancer. Trends Cancer. 2018 Jun;4(6):418-428.

35. Le DT, Wang-Gillam A, Picozzi V, Greten TF, Crocenzi T, Springett G, Morse M, Zeh H, Cohen D, Fine RL, Onners B, Uram JN, Laheru DA, Lutz ER, Solt S, Murphy AL, Skoble J, Lemmens E, Grous J, Dubensky T Jr, Brockstedt DG, Jaffee EM. Safety and survival with GVAX pancreas prime and Listeria Monocytogenes-expressing mesothelin (CRS-207) boost vaccines for metastatic pancreatic cancer. J Clin Oncol. 2015 Apr 20;33(12):1325-33.

36. Bernhardt SL, Gjertsen MK, Trachsel S, Møller M, Eriksen JA, Meo M, Buanes T, Gaudernack G. Telomerase peptide vaccination of patients with non-resectable pancreatic cancer: A dose escalating phase I/II study. Br J Cancer. 2006 Dec 4;95(11):1474-82.

37. Mayanagi S, Kitago M, Sakurai T, Matsuda T, Fujita T, Higuchi H, Taguchi J, Takeuchi H, Itano O, Aiura K, Hamamoto Y, Takaishi H, Okamoto M, Sunamura M, Kawakami Y, Kitagawa Y. Phase I pilot study of Wilms tumor gene 1 peptide-pulsed dendritic cell vaccination combined with gemcitabine in pancreatic cancer. Cancer Sci. 2015 Apr;106(4):397-406.

38. Hewitt DB, Nissen N, Hatoum H, Musher B, Seng J, Coveler AL, Al-Rajabi R, Yeo CJ, Leiby B, Banks J, Balducci L, Vaccaro G, LoConte N, George TJ, Brenner W, Elquza E, Vahanian N, Rossi G, Kennedy E, Link C, Lavu H. A Phase 3 Randomized Clinical Trial of Chemotherapy With or Without Algenpantucel-L (HyperAcute-Pancreas) Immunotherapy in Subjects With Borderline Resectable or Locally Advanced Unresectable Pancreatic Cancer. Ann Surg. 2022 Jan 1;275(1):45-53.

39. Middleton G, Silcocks P, Cox T, Valle J, Wadsley J, Propper D, Coxon F, Ross P, Madhusudan S, Roques T, Cunningham D, Falk S, Wadd N, Harrison M, Corrie P, Iveson T, Robinson A, McAdam K, Eatock M, Evans J, Archer C, Hickish T, Garcia-Alonso A, Nicolson M, Steward W, Anthoney A, Greenhalf W, Shaw V, Costello E, Naisbitt D, Rawcliffe C, Nanson G, Neoptolemos J. Gemcitabine and capecitabine with or without telomerase peptide vaccine GV1001 in patients with locally advanced or metastatic pancreatic cancer (TeloVac): an open-label, randomised, phase 3 trial. Lancet Oncol. 2014 Jul;15(8):829-40.

40. Tsujikawa T, Crocenzi T, Durham JN, Sugar EA, Wu AA, Onners B, Nauroth JM, Anders RA, Fertig EJ, Laheru DA, Reiss K, Vonderheide RH, Ko AH, Tempero MA, Fisher GA, Considine M, Danilova L, Brockstedt DG, Coussens LM, Jaffee EM, Le DT. Evaluation of Cyclophosphamide/GVAX Pancreas Followed by Listeria-Mesothelin (CRS-207) with or without Nivolumab in Patients with Pancreatic Cancer. Clin Cancer Res. 2020 Jul 15;26(14):3578-3588.

41. Kochenderfer JN, Wilson WH, Janik JE, Dudley ME, Stetler-Stevenson M, Feldman SA, Maric I, Raffeld M, Nathan DA, Lanier BJ, Morgan RA, Rosenberg SA. Eradication of B-lineage cells and regression of lymphoma in a patient treated with autologous T cells genetically engineered to recognize CD19. Blood. 2010 Nov 18;116(20):4099-102.

42. Porter DL, Levine BL, Kalos M, Bagg A, June CH. Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. N Engl J Med. 2011 Aug 25;365(8):725-33. doi: 10.1056/NEJMoa1103849. Epub 2011 Aug 10. Erratum in: N Engl J Med. 2016 Mar 10;374(10):998.

43. Raj D, Yang MH, Rodgers D, Hampton EN, Begum J, Mustafa A, Lorizio D, Garces I, Propper D, Kench JG, Kocher HM, Young TS, Aicher A, Heeschen C. Switchable CAR-T cells mediate remission in metastatic pancreatic ductal adenocarcinoma. Gut. 2019 Jun;68(6):1052-1064.

44. Pardoll DM, Topalian SL. The role of $CD4^+$ T cell responses in antitumor immunity. Curr Opin Immunol. 1998 Oct;10(5):588-94.

45. Liu X, Hogg GD, DeNardo DG. Rethinking immune checkpoint blockade: 'Beyond the T cell'. J Immunother Cancer. 2021 Jan;9(1):e001460.

46. O'Neill RE, Cao X. Co-stimulatory and co-inhibitory pathways in cancer immunotherapy. Adv Cancer Res. 2019;143:145-194.

47. Zhang H, Dai Z, Wu W, Wang Z, Zhang N, Zhang L, Zeng WJ, Liu Z, Cheng Q. Regulatory mechanisms of immune checkpoints PD-L1 and CTLA-4 in cancer. J Exp Clin Cancer Res. 2021 Jun 4;40(1):184.

48. Rotte A. Combination of CTLA-4 and PD-1 blockers for treatment of cancer. J Exp Clin Cancer Res. 2019 Jun 13;38(1):255.

49. Wojtukiewicz MZ, Rek MM, Karpowicz K, Górska M, Polityńska B, Wojtukiewicz AM, Moniuszko M, Radziwon P, Tucker SC, Honn KV. Inhibitors of immune checkpoints-PD-1, PD-L1, CTLA-4-new opportunities for cancer patients and a new challenge for internists and general practitioners. Cancer Metastasis Rev. 2021 Sep;40(3):949-982.

50. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, Powderly

JD, Sosman JA, Atkins MB, Leming PD, Spigel DR, Antonia SJ, Drilon A, Wolchok JD, Carvajal RD, McHenry MB, Hosein F, Harbison CT, Grosso JF, Sznol M. Five-Year Survival and Correlates Among Patients With Advanced Melanoma, Renal Cell Carcinoma, or Non-Small Cell Lung Cancer Treated With Nivolumab. JAMA Oncol. 2019 Oct 1;5(10):1411-1420.

51. Henriksen A, Dyhl-Polk A, Chen I, Nielsen D. Checkpoint inhibitors in pancreatic cancer. Cancer Treat Rev. 2019 Aug;78:17-30.

52. O'Reilly EM, Oh DY, Dhani N, Renouf DJ, Lee MA, Sun W, Fisher G, Hezel A, Chang SC, Vlahovic G, Takahashi O, Yang Y, Fitts D, Philip PA. Durvalumab With or Without Tremelimumab for Patients With Metastatic Pancreatic Ductal Adenocarcinoma: A Phase 2 Randomized Clinical Trial. JAMA Oncol. 2019 Oct 1;5(10):1431-1438.

53. Xu Z, Jin B. A novel interface consisting of homologous immunoglobulin superfamily members with multiple functions. Cell Mol Immunol. 2010 Jan;7(1):11-9.

54. Wang PL, O'Farrell S, Clayberger C, Krensky AM. Identification and molecular cloning of tactile. A novel human T cell activation antigen that is a member of the Ig gene superfamily. J Immunol. 1992 Apr 15;148(8):2600-8.

55. Georgiev H, Ravens I, Papadogianni G, Bernhardt G. Coming of Age: CD96 Emerges as Modulator of Immune Responses. Front Immunol. 2018 May 17;9:1072.

56. Meyer D, Seth S, Albrecht J, Maier MK, du Pasquier L, Ravens I, Dreyer L, Burger R, Gramatzki M, Schwinzer R, Kremmer E, Foerster R, Bernhardt G. CD96 interaction with CD155 via its first Ig-like domain is modulated by alternative splicing or mutations in distal Ig-like domains. J Biol Chem. 2009 Jan 23;284(4):2235-44.

57. Dougall WC, Kurtulus S, Smyth MJ, Anderson AC. TIGIT and CD96: new checkpoint receptor targets for cancer immunotherapy. Immunol Rev. 2017 Mar;276(1):112-120.

58. Feng S, Isayev O, Werner J, Bazhin AV. CD96 as a Potential Immune Regulator in Cancers. Int J Mol Sci. 2023 Jan 9;24(2):1303.

59. Wong KC, Lin J, Li X, Lin Q, Liang C, Song YQ. Heterodimeric DNA motif synthesis and validations. Nucleic Acids Res. 2019 Feb 28;47(4):1628-1636.

60. Hepkema J, Lee NK, Stewart BJ, Ruangroengkulrith S, Charoensawan V, Clatworthy MR, Hemberg M. Predicting the impact of sequence motifs on gene regulation using single-cell data. Genome Biol. 2023 Aug 15;24(1):189.

61. Lanier LL. Up on the tightrope: natural killer cell activation and inhibition. Nat Immunol. 2008 May;9(5):495-502.

62. Coxon CH, Geer MJ, Senis YA. ITIM receptors: more than just inhibitors of platelet activation. Blood. 2017 Jun 29;129(26):3407-3418.

63. Bruyns E, Marie-Cardine A, Kirchgessner H, Sagolla K, Shevchenko A, Mann M, Autschbach F, Bensussan A, Meuer S, Schraven B. T cell receptor (TCR) interacting molecule (TRIM), a novel disulfide-linked dimer associated with the TCR-CD3-zeta complex, recruits intracellular signaling proteins to the plasma membrane. J Exp Med. 1998 Aug 3;188(3):561-75.

64. Marasco M, Berteotti A, Weyershaeuser J, Thorausch N, Sikorska J, Krausze J, Brandt HJ, Kirkpatrick J, Rios P, Schamel WW, Köhn M, Carlomagno T. Molecular mechanism of SHP2 activation by PD-1 stimulation. Sci Adv. 2020 Jan 31;6(5):eaay4458.

65. Sinclair NR. Immunoreceptor tyrosine-based inhibitory motifs on activating molecules. Crit Rev Immunol. 2000;20(2):89-102.

66. Li M, Xia P, Du Y, Liu S, Huang G, Chen J, Zhang H, Hou N, Cheng X, Zhou L, Li P, Yang X, Fan Z. T-cell immunoglobulin and ITIM domain (TIGIT) receptor/poliovirus receptor (PVR) ligand engagement suppresses interferon-γ production of natural killer cells via β-arrestin 2-mediated negative signaling. J Biol Chem. 2014 Jun 20;289(25):17647-57.

67. Wang CQ, Choy FC, Sanny A, Murakami T, Tan AH, Lam KP. An Inhibitory Role for Human CD96 Endodomain in T Cell Anti-Tumor Responses. Cells. 2023 Jan 13;12(2):309.

68. Zhang H, Liu Q, Lei Y, Zhou J, Jiang W, Cui Y, He Q, Zhu J, Zhu Z, Sun Y, Ke Z. Direct interaction between CD155 and CD96 promotes immunosuppression in lung adenocarcinoma. Cell Mol Immunol. 2021 Jun;18(6):1575-1577.

69. Wang Y, Wang C, Qiu J, Qu X, Peng J, Lu C, Zhang M, Zhang M, Qi X, Li G, Hua K. Targeting CD96 overcomes PD-1 blockade resistance by enhancing $CD8^+$ TIL function in cervical cancer. J Immunother Cancer. 2022 Mar;10(3):e003667.

70. Sun H, Huang Q, Huang M, Wen H, Lin R, Zheng M, Qu K, Li K, Wei H, Xiao W, Sun R, Tian Z, Sun C. Human CD96 Correlates to Natural Killer Cell Exhaustion and Predicts the Prognosis of Human Hepatocellular Carcinoma. Hepatology. 2019 Jul;70(1):168-183.

71. Lepletier A, Lutzky VP, Mittal D, Stannard K, Watkins TS, Ratnatunga CN, Smith C, McGuire HM, Kemp RA, Mukhopadhyay P, Waddell N, Smyth MJ, Dougall WC, Miles JJ. The immune checkpoint CD96 defines a distinct lymphocyte phenotype and is highly expressed on tumor-infiltrating T cells. Immunol Cell Biol. 2019 Feb;97(2):152-164.

72. Ye W, Luo C, Liu F, Liu Z, Chen F. CD96 Correlates With Immune Infiltration and Impacts Patient Prognosis: A Pan-Cancer Analysis. Front Oncol. 2021 Feb 19;11:634617.

73. Mendelsohn CL, Wimmer E, Racaniello VR. Cellular receptor for poliovirus: molecular

cloning, nucleotide sequence, and expression of a new member of the immunoglobulin superfamily. Cell. 1989 Mar 10;56(5):855-65.

74. He Y, Bowman VD, Mueller S, Bator CM, Bella J, Peng X, Baker TS, Wimmer E, Kuhn RJ, Rossmann MG. Interaction of the poliovirus receptor with poliovirus. Proc Natl Acad Sci U S A. 2000 Jan 4;97(1):79-84.

75. Fuchs A, Colonna M. The role of NK cell recognition of nectin and nectin-like proteins in tumor immunosurveillance. Semin Cancer Biol. 2006 Oct;16(5):359-66.

76. Sakisaka T, Ikeda W, Ogita H, Fujita N, Takai Y. The roles of nectins in cell adhesions: cooperation with other cell adhesion molecules and growth factor receptors. Curr Opin Cell Biol. 2007 Oct;19(5):593-602.

77. Rikitake Y, Mandai K, Takai Y. The role of nectins in different types of cell-cell adhesion. J Cell Sci. 2012 Aug 15;125(Pt 16):3713-22.

78. Masson D, Jarry A, Baury B, Blanchardie P, Laboisse C, Lustenberger P, Denis MG. Overexpression of the CD155 gene in human colorectal carcinoma. Gut. 2001 Aug;49(2):236-40.

79. Nakai R, Maniwa Y, Tanaka Y, Nishio W, Yoshimura M, Okita Y, Ohbayashi C, Satoh N, Ogita H, Takai Y, Hayashi Y. Overexpression of Necl-5 correlates with unfavorable prognosis in patients with lung adenocarcinoma. Cancer Sci. 2010 May;101(5):1326-30.

80. Bevelacqua V, Bevelacqua Y, Candido S, Skarmoutsou E, Amoroso A, Guarneri C, Strazzanti A, Gangemi P, Mazzarino MC, D'Amico F, McCubrey JA, Libra M, Malaponte G. Nectin like-5 overexpression correlates with the malignant phenotype in cutaneous melanoma. Oncotarget. 2012 Aug;3(8):882-92.

81. Nishiwada S, Sho M, Yasuda S, Shimada K, Yamato I, Akahori T, Kinoshita S, Nagai M, Konishi N, Nakajima Y. Clinical significance of CD155 expression in human pancreatic cancer. Anticancer Res. 2015 Apr;35(4):2287-97. Erratum in: Anticancer Res. 2015 Jul;35(7):4371.

82. Sloan KE, Eustace BK, Stewart JK, Zehetmeier C, Torella C, Simeone M, Roy JE, Unger C, Louis DN, Ilag LL, Jay DG. CD155/PVR plays a key role in cell motility during tumor cell invasion and migration. BMC Cancer. 2004 Oct 7;4:73.

83. Yu X, Harden K, Gonzalez LC, Francesco M, Chiang E, Irving B, Tom I, Ivelja S, Refino CJ, Clark H, Eaton D, Grogan JL. The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells. Nat Immunol. 2009 Jan;10(1):48-57.

84. Boles KS, Vermi W, Facchetti F, Fuchs A, Wilson TJ, Diacovo TG, Cella M, Colonna

M. A novel molecular interaction for the adhesion of follicular CD4 T cells to follicular DC. Eur J Immunol. 2009 Mar;39(3):695-703.

85. Levin SD, Taft DW, Brandt CS, Bucher C, Howard ED, Chadwick EM, Johnston J, Hammond A, Bontadelli K, Ardourel D, Hebb L, Wolf A, Bukowski TR, Rixon MW, Kuijper JL, Ostrander CD, West JW, Bilsborough J, Fox B, Gao Z, Xu W, Ramsdell F, Blazar BR, Lewis KE. Vstm3 is a member of the CD28 family and an important modulator of T-cell function. Eur J Immunol. 2011 Apr;41(4):902-15.

86. Kojima H, Kanada H, Shimizu S, Kasama E, Shibuya K, Nakauchi H, Nagasawa T, Shibuya A. CD226 mediates platelet and megakaryocytic cell adhesion to vascular endothelial cells. J Biol Chem. 2003 Sep 19;278(38):36748-53.

87. Reymond N, Imbert AM, Devilard E, Fabre S, Chabannon C, Xerri L, Farnarier C, Cantoni C, Bottino C, Moretta A, Dubreuil P, Lopez M. DNAM-1 and PVR regulate monocyte migration through endothelial junctions. J Exp Med. 2004 May 17;199(10):1331-41.

88. Ma D, Sun Y, Lin D, Wang H, Dai B, Zhang X, Ouyang W, Jian J, Jia W, Xu X, Jin B. CD226 is expressed on the megakaryocytic lineage from hematopoietic stem cells/progenitor cells and involved in its polyploidization. Eur J Haematol. 2005 Mar;74(3):228-40.

89. Bachelet I, Munitz A, Mankutad D, Levi-Schaffer F. Mast cell costimulation by CD226/CD112 (DNAM-1/Nectin-2): a novel interface in the allergic process. J Biol Chem. 2006 Sep 15;281(37):27190-6.

90. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. Trends Immunol. 2001 Nov;22(11):633-40.

91. Freud AG, Mundy-Bosse BL, Yu J, Caligiuri MA. The Broad Spectrum of Human Natural Killer Cell Diversity. Immunity. 2017 Nov 21;47(5):820-833.

92. Abel AM, Yang C, Thakar MS, Malarkannan S. Natural Killer Cells: Development, Maturation, and Clinical Utilization. Front Immunol. 2018 Aug 13;9:1869.

93. Gama SM, Varela VA, Ribeiro NM, Bizzarro B, Hernandes C, Aloia TPA, Amano MT, Pereira WO. AKT inhibition interferes with the expression of immune checkpoint proteins and increases NK-induced killing of HL60-AML cells. Einstein (Sao Paulo). 2023 Jun 19;21:eAO0171.

94. Zhao J, Li L, Feng X, Fan X, Yin H, Lu Q. T cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain as a promising immune checkpoint target for the treatment of SLE. Lupus. 2024 Mar;33(3):209-216.

95. Farhangnia P, Akbarpour M, Yazdanifar M, Aref AR, Delbandi AA, Rezaei N. Advances in therapeutic targeting of immune checkpoints receptors within the CD96-TIGIT axis: clinical implications and future perspectives. Expert Rev Clin Immunol. 2022 Dec;18(12):1217-1237.

96. Fuchs A, Cella M, Giurisato E, Shaw AS, Colonna M. Cutting edge: CD96 (tactile) promotes NK cell-target cell adhesion by interacting with the poliovirus receptor (CD155). J Immunol. 2004 Apr 1;172(7):3994-8.

97. Liu L, You X, Han S, Sun Y, Zhang J, Zhang Y. CD155/TIGIT, a novel immune checkpoint in human cancers (Review). Oncol Rep. 2021 Mar;45(3):835-845.

98. Wu B, Zhong C, Lang Q, Liang Z, Zhang Y, Zhao X, Yu Y, Zhang H, Xu F, Tian Y. Poliovirus receptor (PVR)-like protein cosignaling network: new opportunities for cancer immunotherapy. J Exp Clin Cancer Res. 2021 Aug 25;40(1):267.

99. Chiang EY, de Almeida PE, de Almeida Nagata DE, Bowles KH, Du X, Chitre AS, Banta KL, Kwon Y, McKenzie B, Mittman S, Cubas R, Anderson KR, Warming S, Grogan JL. CD96 functions as a co-stimulatory receptor to enhance $CD8^+$ T cell activation and effector responses. Eur J Immunol. 2020 Jun;50(6):891-902.

100. Mittal D, Lepletier A, Madore J, Aguilera AR, Stannard K, Blake SJ, Whitehall VLJ, Liu C, Bettington ML, Takeda K, Long GV, Scolyer RA, Lan R, Siemers N, Korman A, Teng MWL, Johnston RJ, Dougall WC, Smyth MJ. CD96 Is an Immune Checkpoint That Regulates $CD8^+$ T-cell Antitumor Function. Cancer Immunol Res. 2019 Apr;7(4):559-571.

101. Delmastro-Greenwood MM, Piganelli JD. Changing the energy of an immune response. Am J Clin Exp Immunol. 2013 Feb 27;2(1):30-54.

102. Pearce EL, Pearce EJ. Metabolic pathways in immune cell activation and quiescence. Immunity. 2013 Apr 18;38(4):633-43.

103. Pearce EL, Poffenberger MC, Chang CH, Jones RG. Fueling immunity: insights into metabolism and lymphocyte function. Science. 2013 Oct 11;342(6155):1242454.

104. El Sayed R, Haibe Y, Amhaz G, Bouferraa Y, Shamseddine A. Metabolic Factors Affecting Tumor Immunogenicity: What Is Happening at the Cellular Level? Int J Mol Sci. 2021 Feb 21;22(4):2142.

105. Faas MM, de Vos P. Mitochondrial function in immune cells in health and disease. Biochim Biophys Acta Mol Basis Dis. 2020 Oct 1;1866(10):165845.

106. Zhang C, Liu Y. Targeting NK Cell Checkpoint Receptors or Molecules for Cancer Immunotherapy. Front Immunol. 2020 Jun 23;11:1295.

107. Hargadon KM, Johnson CE, Williams CJ. Immune checkpoint blockade therapy for cancer: An overview of FDA-approved immune checkpoint inhibitors. Int Immunopharmacol. 2018 Sep;62:29-39.

108. Stanko K, Iwert C, Appelt C, Vogt K, Schumann J, Strunk FJ, Ahrlich S, Schlickeiser S, Romagnani C, Jürchott K, Meisel C, Willimsky G, Kühl AA, Sawitzki B. CD96 expression determines the inflammatory potential of IL-9-producing Th9 cells. Proc Natl Acad Sci U S A. 2018 Mar 27;115(13):E2940-E2949.

109. Harjunpää H, Guillerey C. TIGIT as an emerging immune checkpoint. Clin Exp Immunol. 2020 May;200(2):108-119.

110. Wang F, Hou H, Wu S, Tang Q, Liu W, Huang M, Yin B, Huang J, Mao L, Lu Y, Sun Z. TIGIT expression levels on human NK cells correlate with functional heterogeneity among healthy individuals. Eur J Immunol. 2015 Oct;45(10):2886-97.

111. Vo AV, Takenaka E, Shibuya A, Shibuya K. Expression of DNAM-1 (CD226) on inflammatory monocytes. Mol Immunol. 2016 Jan;69:70-6.

112. Tahara-Hanaoka S, Shibuya K, Onoda Y, Zhang H, Yamazaki S, Miyamoto A, Honda S, Lanier LL, Shibuya A. Functional characterization of DNAM-1 (CD226) interaction with its ligands PVR (CD155) and nectin-2 (PRR-2/CD112). Int Immunol. 2004 Apr;16(4):533-8.

113. Belizário JE, Neyra JM, Setúbal Destro Rodrigues MF. When and how NK cell-induced programmed cell death benefits immunological protection against intracellular pathogen infection. Innate Immun. 2018 Nov;24(8):452-465.

114. Huang Z, Qi G, Miller JS, Zheng SG. CD226: An Emerging Role in Immunologic Diseases. Front Cell Dev Biol. 2020 Jul 24;8:564.

115. Chan CJ, Martinet L, Gilfillan S, Souza-Fonseca-Guimaraes F, Chow MT, Town L, Ritchie DS, Colonna M, Andrews DM, Smyth MJ. The receptors CD96 and CD226 oppose each other in the regulation of natural killer cell functions. Nat Immunol. 2014 May;15(5):431-8.

116. Zahm CD, Colluru VT, McIlwain SJ, Ong IM, McNeel DG. TLR Stimulation during T-cell Activation Lowers PD-1 Expression on $CD8^+$ T Cells. Cancer Immunol Res. 2018 Nov;6(11):1364-1374.

117. Nakanishi T, Yoneshima Y, Okamura K, Yanagihara T, Hashisako M, Iwasaki T, Haratake N, Mizusaki S, Ota K, Iwama E, Takenaka T, Tanaka K, Yoshizumi T, Oda Y, Okamoto I. MicroRNA-326 negatively regulates CD155 expression in lung adenocarcinoma. Cancer Sci. 2023 Oct;114(10):4101-4113.

118. Liang J, Liao L, Xie L, Tang W, Yu X, Lu Y, Chen H, Xu J, Sun L, Wu H, Cui C, Tan Y. PITPNC1 Suppress $CD8^+$ T cell immune function and promote radioresistance in rectal cancer by modulating FASN/CD155. J Transl Med. 2024 Jan 30;22(1):117.

119. He W, Zhang H, Han F, Chen X, Lin R, Wang W, Qiu H, Zhuang Z, Liao Q, Zhang W, Cai Q, Cui Y, Jiang W, Wang H, Ke Z. CD155T/TIGIT Signaling Regulates $CD8^+$ T-cell Metabolism and Promotes Tumor Progression in Human Gastric Cancer. Cancer Res. 2017 Nov 15;77(22):6375-6388.

120. Ogando J, Sáez ME, Santos J, Nuevo-Tapioles C, Gut M, Esteve-Codina A, Heath S, González-Pérez A, Cuezva JM, Lacalle RA, Mañes S. PD-1 signaling affects cristae morphology and leads to mitochondrial dysfunction in human $CD8^+$ T lymphocytes. J Immunother Cancer. 2019 Jun 13;7(1):151.

121. Peng YP, Xi CH, Zhu Y, Yin LD, Wei JS, Zhang JJ, Liu XC, Guo S, Fu Y, Miao Y. Altered expression of CD226 and CD96 on natural killer cells in patients with pancreatic cancer. Oncotarget. 2016 Oct 11;7(41):66586-66594.

122. Yong H, Cheng R, Li X, Gao G, Jiang X, Cheng H, Zhou X, Zhao W. CD155 expression and its prognostic value in postoperative patients with breast cancer. Biomed Pharmacother. 2019 Jul;115:108884.

123. Zhu X, Liang R, Lan T, Ding D, Huang S, Shao J, Zheng Z, Chen T, Huang Y, Liu J, Pathak JL, Wei H, Wei B. Tumor-associated macrophage-specific CD155 contributes to M2-phenotype transition, immunosuppression, and tumor progression in colorectal cancer. J Immunother Cancer. 2022 Sep;10(9):e004219.

124. Zhang Q, Zhong H, Fan Y, Liu Q, Song J, Yao S, Cao F. Immune and Clinical Features of CD96 Expression in Glioma by in silico Analysis. Front Bioeng Biotechnol. 2020 Jun 30;8:592.

125. Freed-Pastor WA, Lambert LJ, Ely ZA, Pattada NB, Bhutkar A, Eng G, Mercer KL, Garcia AP, Lin L, Rideout WM 3rd, Hwang WL, Schenkel JM, Jaeger AM, Bronson RT, Westcott PMK, Hether TD, Divakar P, Reeves JW, Deshpande V, Delorey T, Phillips D, Yilmaz OH, Regev A, Jacks T. The CD155/TIGIT axis promotes and maintains immune evasion in neoantigen-expressing pancreatic cancer. Cancer Cell. 2021 Oct 11;39(10):1342-1360.e14.

126. Ma H, Chen X, Mo S, Mao X, Chen J, Liu Y, Lu Z, Yu S, Chen J. The spatial coexistence of TIGIT/CD155 defines poorer survival and resistance to adjuvant chemotherapy in pancreatic ductal adenocarcinoma. Theranostics. 2023 Aug 18;13(13):4601-4614.

127. Xu C, Fang H, Gu Y, Yu K, Wang J, Lin C, Zhang H, Li H, He H, Liu H, Li R. Impact of intratumoural CD96 expression on clinical outcome and therapeutic benefit in gastric cancer. Cancer Sci. 2022 Dec;113(12):4070-4081.

128. Liao X, Huang K, Huang R, Liu X, Han C, Yu L, Yu T, Yang C, Wang X, Peng T. Genome-scale analysis to identify prognostic markers in patients with early-stage pancreatic ductal adenocarcinoma after pancreaticoduodenectomy. Onco Targets Ther. 2017 Sep 12;10:4493-4506.

129. Bhalla S, Chaudhary K, Kumar R, Sehgal M, Kaur H, Sharma S, Raghava GP. Gene expression-based biomarkers for discriminating early and late stage of clear cell renal cancer. Sci Rep. 2017 Mar 28;7:44997.

130. Bédard P, Gauvin S, Ferland K, Caneparo C, Pellerin È, Chabaud S, Bolduc S. Innovative Human Three-Dimensional Tissue-Engineered Models as an Alternative to Animal Testing. Bioengineering (Basel). 2020 Sep 17;7(3):115.

131. Barré-Sinoussi F, Montagutelli X. Animal models are essential to biological research: issues and perspectives. Future Sci OA. 2015 Nov 1;1(4):FSO63.

132. Domínguez-Oliva A, Hernández-Ávalos I, Martínez-Burnes J, Olmos-Hernández A, Verduzco-Mendoza A, Mota-Rojas D. The Importance of Animal Models in Biomedical Research: Current Insights and Applications. Animals (Basel). 2023 Mar 31;13(7):1223.

133. Businaro L, De Ninno A, Schiavoni G, Lucarini V, Ciasca G, Gerardino A, Belardelli F, Gabriele L, Mattei F. Cross talk between cancer and immune cells: exploring complex dynamics in a microfluidic environment. Lab Chip. 2013 Jan 21;13(2):229-39.

134. Veninga V, Voest EE. Tumor organoids: Opportunities and challenges to guide precision medicine. Cancer Cell. 2021 Sep 13;39(9):1190-1201.

135. Xu H, Jiao D, Liu A, Wu K. Tumor organoids: applications in cancer modeling and potentials in precision medicine. J Hematol Oncol. 2022 May 12;15(1):58.

136. Paolini R, Molfetta R. CD155 and Its Receptors as Targets for Cancer Therapy. Int J Mol Sci. 2023 Aug 19;24(16):12958.

137. Zhang H, Yang Z, Du G, Cao L, Tan B. CD155-Prognostic and Immunotherapeutic Implications Based on Multiple Analyses of Databases Across 33 Human Cancers. Technol Cancer Res Treat. 2021 Jan-Dec;20:1533033820980088.

138. Meric-Bernstam F, Larkin J, Tabernero J, Bonini C. Enhancing anti-tumour efficacy with immunotherapy combinations. Lancet. 2021 Mar 13;397(10278):1010-1022.

139. Yang Y. Cancer immunotherapy: harnessing the immune system to battle cancer. J Clin

Invest. 2015 Sep;125(9):3335-7.

140. Yi M, Zheng X, Niu M, Zhu S, Ge H, Wu K. Combination strategies with PD-1/PD-L1 blockade: current advances and future directions. Mol Cancer. 2022 Jan 21;21(1):28.

141. Yap TA, Parkes EE, Peng W, Moyers JT, Curran MA, Tawbi HA. Development of Immunotherapy Combination Strategies in Cancer. Cancer Discov. 2021 Jun;11(6):1368-1397.

142. Li J, Xia Q, Di C, Li C, Si H, Zhou B, Yu S, Li Y, Huang J, Lu Y, Huang M, Liang H, Liu X, Zhao Q. Tumor Cell-Intrinsic CD96 Mediates Chemoresistance and Cancer Stemness by Regulating Mitochondrial Fatty Acid β-Oxidation. Adv Sci (Weinh). 2023 Mar;10(7):e2202956.

Acknowledgements

As my time at the General, Visceral, and Transplant Surgery department of Großhadern Hospital of Ludwig Maximilian University of Munich is ending, looking back, from distant China to Germany, everything has been completely new - the environment, the language, the things, the colleagues, all so novel. In the blink of an eye, three years are about to pass. I still vividly remember taking off from Chongqing on October 15, 2021, transferring in Guangzhou and Amsterdam, and finally arriving at Dusseldorf International Airport. Due to the time difference, I fell asleep around five in the afternoon and woke up in the middle of the night around three. At that moment, I still couldn't believe I had arrived at the other end of the Earth to embark on a brand-new learning journey. And so, three years have flown by. I still remember setting myself three goals before coming to Germany: completing my studies, improving my English, and sticking to a fitness routine. Now it seems I've only achieved the first two goals; the third one will have to wait for the future.

What I most want to talk about is the people I have encountered in Germany who have been very important to me. In the beginning, I'd like to thank my supervisor, Professor Dr. Alexander Bazhin. I remember the first interview vividly; Professor Bazhin's friendly demeanor and sincere words made me feel immediately at ease. He invited me to visit the laboratory in Munich for a face-to-face discussion, during which he provided a detailed introduction to the laboratory facilities and equipment in each room. He also introduced me to some Chinese students in the laboratory to help me better acquaint myself with the environment. After the interview, Professor Bazhin and I had dinner together, during which we engaged in deep conversations about life, research, and more. This series of interactions made me feel a warmth in Germany that I had never experienced before and strengthened my desire to join Professor Bazhin's laboratory. Upon entering the laboratory, Professor Bazhin provided me with abundant scientific guidance and advice, ranging from research planning to experimental operations to article writing, tailored

to my project, which enabled my research to progress smoothly. During leisure time, Professor Bazhin and I also shared small aspects of our lives with each other. In summary, Professor Bazhin is not only my supervisor but also feels like a friend to me. I am grateful for his advice throughout my MD studies.

Next, I want to convey my appreciation to the laboratory technician, Gesse Nadine. When I first entered the laboratory, Nadine familiarized me with the lab and taught me some basic experimental skills, such as cell culture and how to operate FACS analysis, among others. She also patiently answered many questions I had during experiments. I am very thankful for Nadine's patience and professional expertise, which helped me quickly adapt to the laboratory environment and kick-start my project.

Additionally, I want to thank our laboratory secretary, Ms. Krause Elvira. Ms. Elvira helped me with various tasks, including enrollment registration and purchasing reagents, allowing me to focus more smoothly on my research.

I'd additionally appreciate it to everyone who works at the General, Visceral, and Transplant Surgery department of Großhadern Hospital. Thank you for your assistance in the laboratory and for sharing many happy moments with me during leisure time. Without you, my doctoral journey would not have been as enriching and colorful. Thank you.

I intend to express gratitude to my parents, elder aunt, elder aunt’s husband, cousin, cousin's husband, and Aunt Zhaoling You. Without your encouragement and support, I would not have achieved what I have today.

I am also thankful for the funding from the China Scholarship Council (Grant No. 202106370047), which relieved me of financial pressure and allowed me to focus on research in Munich.

With the completion of the industrial and information revolutions, people's lives have undergone tremendous changes in the past one or two centuries, with quality of life and longevity reaching historic highs. However, disruptions to daily routines due to faster-paced lifestyles, environmental

pollution, dietary health, genetic factors, and other issues have led to cancer, causing many people to lose their lives and bringing immense grief to their families. Yet, for many malignant cancers, apart from early surgical removal, there aren't many effective treatments available that completely eradicate cancer. Often, the side effects severely impact patients' quality of life. This is a major challenge facing human health today.

I am fortunate to be involved in the field of cancer immunotherapy. I believe that immunotherapy holds the most promise for curing cancer. However, there are still many challenges to overcome. I am willing to work together with colleagues around the world to contribute my efforts to the conquest of cancer for humanity.

Affidavit

Feng, Shikai

Surname, first name

I hereby declare, that the submitted thesis entitled

CD96 as a potential immunotherapy target for pancreatic cancer

is my own work. I have only used the sources indicated and have not made unauthorised use of services of a third party. Where the work of others has been quoted or reproduced, the source is always given.

I further declare that the dissertation presented here has not been submitted in the same or similar form to any other institution for the purpose of obtaining an academic degree.

Changsha, 22.10.2025

Place, Date

Shikai Feng

Signature doctoral candidate